STAT

The Effect of Low Temperatures on the Development of Various Microbes, Especially on the Development of the Acid-Fast Bacillus Tuberculosis. First Report: The Effect of Low Temperatures on the Development of Various Microbes Besides Acid-Fast Microbes

HOKKAIDÔ IGAKU ZASSHI [Hokkaido Medical Journal] 17 (3) [illegible]
TOZUKA Iwataro, Hokkaido, March 1939

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INSERT: [illegible] (1893), Kent (1894), Kruschinski (1915)

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(1898), [illegible] (1901), [illegible] (1902), [illegible] (1903), [illegible] (1904), [illegible] (1906), [illegible] (1907), on Bacillus pestis; [illegible] (1897), [illegible] (1900), [illegible] (1901), Park (1901), [illegible] (1903), [illegible] (1905), [illegible] (1908), Hara (1928), Riku (1930), and Watanabe (1930), on Bacillus dysentriae; [illegible] (1897), Cadeac and [illegible] (1892), [illegible] (1900), and Schope (1926) on the Tuberculosis bacillus; Klepzoff (1895) and Delli (1908) on Bacillus anthracis; Onorato

*Note: Mycobacterium tuberculosis

(1902) on the influenza bacillus; Deisner, Saul and Krall (1907), and Arai (1931) on staphylococcus; and Kisser on Treponema pallidum (Spirochaeta pallida).

Of course, there were variations in duration time of subjection to low temperatures for various degrees of concentration reduction, differences in media, and various species, and for various other conditions, but all experiments agree that bacteria can retain their viability to an extent under low temperature for a fairly long period of time.

The virulence of bacteria is not greatly affected by low temperatures; there were variations depending for a long period. This has been proved by Kuntz and Kent (1917) on spores of Bacillus anthracis; by Kitamoto (1930) on streptococcus; and by F. Botschilich and Sokova (1936) on Bacillus cholerae. However, Sasai (1940) stated that virulence will not be affected even after Bacillus cholerae, Bacillus typhosus, Bacillus diphtheriae, and Bacillus coli are exposed alternately to plus 50 degrees Centigrade and minus 70 degrees Centigrade. Gerrard (1947) stated that the agglutination reaction is not lost after exposing Bacillus typhosus to the low temperature of minus 15 degrees for 12 hours.

It was Tait (1899) who conducted the experiment on the resistance exhibited to extreme low temperature brought about by utilizing liquid air (minus 183 degrees Centigrade). Then Pachehren (1900) experimented with Bacillus typhosus, Bacillus colon, Bacillus diphtheriae, Bacillus cholerae, Bacillus proteus vulgaris, and Lactic acid bacilli by exposing them to liquid air for 20 hours. He proved that these bacteria could still survive under those conditions. Then Ravenel (1900) proved in his experiments that liquid air had little effect on Bacillus anthracis and Staphylococcus. Belli (1902) further proved that this was the case with Bacillus anthracis and its spores.

But, nevertheless, one defect has always accompanied the above stated experiments, which is the fact that they were conducted under the varying conditions of outside temperatures. Temperatures fluctuate each day and the experiments could not be conducted under conditions with a certain uniform low temperature over a given period of time.

Since a constant low temperature laboratory is available in this university, it is possible to maintain in the laboratory a desired low temperature (from minus 30 to minus 50 degrees Centigrade) which simulates, to all extents and purposes, the natural environment. With this equipment an land experiments were conducted on the resistance of various alloyes to low temperature and some of the results noted are presented in this report.

II EXPERIMENTAL PROCEDURE

1. Bacteria Employed in the Experiment

The following microorganisms were utilized for experimental purposes in this study: Staphylococcus (albus, citreus, and aureus), Bacillus coli (cultured from the urines of patients suffering from the Bacillus coli type of cystitis), Bacillus proteus vulgaris, Bacillus pyocyaneus, Bacillus typhosus, Bacillus paratyphosus A, Bacillus paratyphosis B, Bacillus dysenteriae (Shiga-Kruse), Bacillus dysentriae Kongrove A [Japanese classification], Bacillus dysentriae Kongrove B, Bacillus dysenteriae infantum (Bacillus chara-kiota or Sonne-Kruse Bacillus), and Diplococcus pneumoniae. The total types of bacteria numbered twelve. For these experiments, bacteria cultured in ordinary agar slant at 37 degrees Centigrade for 24 hours were used. In the case of Diplococcus pneumoniae, it was cultured on beef-blood agar slants.

2. Low Temperature Apparatus

A constant low temperature laboratory newly constructed in this University in 1956 was used. Experiments were conducted by exposing the above stated cultured bacteria to low temperatures ranging from minus 27 degrees Centigrade to minus 33 degrees Centigrade for a specific time period.

3. Operational Low Temperature Methods

Experiment No 1

Each selected bacteria which had been cultured on an ordinary agar slant (or beef-blood agar slant) at 37 degrees Centigrade for 24 hours was placed in the low temperature laboratory. They were then removed from the low temperature laboratory on the 1st, 4th, 7th, 21st, 35th, 49th, and 70th day. A single loopful of cultured bacteria was removed on each occasion. 5.0 cubic centimeters of sterile saline solution was

then used to make bacterial suspensions. 1.0 cubic centimeter of sterile saline solution was placed in each of 3 tubes set in a test tube rack. Following the successive dilution method, 0.05 cubic centimeter of bacterial suspension were diluted each into the rack of test tubes containing the 1.0 cubic centimeter of sterile saline solution. The diluted bacterial suspension of the 3rd test tube was 8000 times that of the first bacterial suspension. Following this, 0.05 cubic centimeter of each of these diluted bacterial suspensions were separated into 3 petri dishes. Agar was then added and mixed to this to make culture plates. After 24 hours of incubation, the bacterial colony growth was counted with a Jefferson Counting Plate. The method was adopted whereby the average of colony counts in 3 petri dishes was accepted as the obtained results.

One departure from this procedure was the use of blood agar slants for Diplococcus pneumonia cultures. Also carried along in the experiment were contrasting agar plates or control plates of 0.05 cubic centimeter of sterile saline solution. It was made certain that no contaminating colony growth was able to exist on the latter plates.

When frozen ordinary agar slants are removed from the low temperature laboratory and placed at room temperature, within a short time the layer of thin ice becomes detached and melts gradually with the bacteria from the surface of the culture media. During this process the thin ice film starts to melt from the periphery to the center. The substance of the culture media also begins to gradually melt, and the accumulated water remains at the bottom of the tube. After a long time, the bacterial mass on the slant begins to flow down and mix with the water in the bottom of the tube. Under such circumstances, a loopful of bacteria would be removed under entirely different conditions than those undergone by

the control plate. Thus we might have been misled. Therefore, when it was necessary to take a loopful of bacteria, the frozen bacteria were separated with a platinum wire loop before the thin ice film on the culture media began to melt and detach itself from the media. Then one loopful of bacteria was very carefully taken and its contaminants was ascertained by Witt's method.

As far as the control agar plates were concerned, they were always paraffined to avoid contamination and to protect the cultured bacteria from drying out. But it was placed at room temperature during the experiment, one loopful was always taken in the same manner as with the low temperature cultures. Also, similar culture methods were employed. The nutrient bacterial plates were sterile, the agar used and compared with the water produced by the bacterial cultures which represent the low temperature experiments.

The contaminants required about 48 hours to reproduce because they were very even when they were paraffined. After 3 or 4 weeks, the colonies began to lose their substance, and at around 10 weeks the it was difficult to pick out a loopful. For such experiments, it was necessary to suspend saline solution in the plate and then loop to detach the bacteria on the surface of the culture media before one attempted to obtain a loopful.

Experiment No. 2

A few loopfuls of the above stated bacteria cultured on ordinary agar slant at 37 degrees Centigrade for 24 hours was taken and mixed with 100 cubic centimeters of saline solution to make evenly distributed bacterial suspensions. Then 2.0 cubic centimeters of the suspended bacteria were placed in a few test tubes and exposed to low temperature. When they were placed in the low temperature laboratory, the bacterial

susceptible abruptly drops [illegible] the adverse influence of the exposure [illegible]. Since [illegible] solid test tubes were used, little possibility was [illegible] that they would break under the influence of freezing. [illegible] test [illegible] were exposed to [illegible] exposure [illegible] 1, 7, 9, 4, 7, 14, 21, 28, 35, 42, 70, 84, and 105 days. They were then left [illegible] at room temperature (18-26 degrees Centigrade) until the fro[illegible] were thawed [illegible] values. Following this, 0.5 cc [illegible] were transferred to 3 separate solid dishes, mixed with agar, and placed in incubation for 24 hours. Finally the colonies produced were counted [illegible] Quantitative Counting Plate, and the average of the number of colonies [illegible] 3 solid plates was recorded as the experimental result. [illegible] were [illegible] individual [illegible] and the [illegible] indicated to be [illegible] colonies [illegible] contaminating [illegible] colony [illegible] on the fresh plates.

With regard to the controls, the test tubes containing bacterial suspensions were cotton plugged and paraffined to prevent evaporation, and they were always exposed to only room temperature.

Of course, saline solutions, tools, and operations were handled under absolutely sterile conditions.

III RESULTS

Exp. 1. The Effect of low temperature on Bacillus coli, Bacillus Proteus, and Bacillus Proteus Vulgaris

Bacillus coli is a bacteria with a greater capacity to resist various environmental influences than Bacillus typhosus. It is said that they can easily survive a half year of dryness. They also exhibit a high capacity to resist low temperature.

Sage and Stephan (1909) found, in their study on the resistance capacity of Bacillus coli and Bacillus typhosus, that 90-95 percent of either organism died after a 24 hour exposure in water at 22 degrees centigrade, and that only a few of the bacteria which survived were able to decline their cultures and resistance to lack of air. They also found that 50 percent of the Bacilli coli died after 15 minutes of exposure to ice, 95 percent were dead within an hour, and 99 percent had died within 24 hours, but they also found that a few survived even after a 3 months exposure period. Also, Defalton (1906) found that all Bacillus coli, Bacillus typhosus, Streptococcus pyogenes, and Gonococcus bacteria which had been exposed to liquid air at minus 190 degrees Centigrade were able to maintain themselves for 16 months and that all of them flourished in culture media.

In our country, Hiku experimented on the resistance capacity of Bacillus coli, Bacillus typhosus, Bacillus cholerae, and Bacillus dysenteriae to low temperature employing the simple outdoor temperature prevalent in Mukden during the extremely cold season of 1927 and 1928. He proved that Bacillus coli survived after 100 days exposure in sterile city-water and for more than 100 days when exposed in stool and urine. Both these experiments were conducted at temperatures ranging from minus 29 to plus 10 degrees Centigrade. They also found that those attached

to dry clothes survived even after 70 days exposure to outdoor temperatures.

Arai (1931) conducted an experiment regarding the survival rate and the cold-resistance capacity of ordinary Bacillus coloni placed in a refrigerator with the temperature ranging between minus 6 and minus 11 degrees Centigrade. He found that their growth was tremendously reduced after 42 days, dropped to 50 percent after 66 days, and to 30 percent after 69 days. After 75 days they stopped growing altogether. Watanabe (1931) utilizing the cold winter climate of Asahikawa city (ranging between minus 30 and minus 5.3 degrees Centigrade), conducted an experiment on the resistance capacity of Bacillus typhosus, Bacillus dysenteriae, and Bacillus coloni. He found that Bacillus coloni survived even after 40 days exposure.

Bacillus pyocyaneus is also a bacteria with a high power of resistance. When Ito's (1922) experiment is studied on the resistance exhibited by Bacillus pyocyaneus, one is able to have some correct idea about its resistance capacity in the natural environment.

Judging from Ito's experiment on resistance, it can be assumed that the resistance capacity of Bacillus pyocyaneus to low temperature will also be very high.

The resistance capacity of Bacillus proteus vulgaris to low temperature is comparatively high. Prudden (1887) stated that when sterile emulsions of Bacillus proteus vulgaris, Staphylococcus citreus, Bacillus typhosus, and other bacteria were frozen due to an outdoor temperature reaching as low as minus 24 degrees Centigrade, a total count of 8300 Bacillus proteus vulgaris per cubic centimeter was reduced to 88 after 18 days and all were dead on the 51st day.

Again, Macfadyen (1904) stated that Bacillus proteus vulgaris,

Bacillus diphtheriae, Bacillus cholerae, and Bacillus coli at first grew fairly well on culture media after being exposed to the liquid air (minus 180 degrees Centigrade) for 20 hours, but then greatly decreased on the 7th day.

The results of these experiments conducted at the low temperature of approximately minus 30 degrees Centigrade are as follows:

Experiment No 1

Bacillus coli, Bacillus pyocyaneus, and Bacillus proteus vulgaris which were cultured on agar slants at 37 degrees Centigrade for 24 hours were placed in the low temperature laboratory and frozen. The results were as follows (see Table 1).

Bacillus coli: Colonies in both the low temperature exposed plates and the control plates decreased to less than ½ by the 2nd day; then, even after reculturing the diluted bacterial suspension on culture plates, up until the third week almost the same number of colonies were counted. But, after the 4th week, the surface of the control plate culture media at room temperature gradually became dry despite the fact that it had been paraffined. At the same time, the number of colonies on the control plate gradually decreased and by the 50th day less than one-fourth of the original remained, and only 1 out of 250 survived by the 70th day.

In contrast, no marked change could be found on those plates which underwent low temperature exposure even after 3 weeks. Roughly, there were as many colonies found on the 70th day as were found on the 2nd day.

Bacillus pyocyaneus: Colony growth at low temperature drastically decreased within a week, but roughly the number of colonies re-

raised the seed up until the 42nd day. After 49 days, they again drastically decreased to less than 10% of the original number. After 70 days, some of them still possessed the ability to survive.

In the case of the control media exposed to room temperature, the number of colonies produced decreased gradually during the first 28 days, but they always exceeded in number those which had been exposed to low temperature. But, after 35 days, the number of colonies being produced on culture plates drastically decreased due to the dryness of the culture media. After that, there were almost as many colonies as those which were able to grow after low temperature exposure. When the degree of pigment formation was checked, no marked difference could be noted for those which had been subjected to low temperature as well as for those subjected only to room temperature. Even after 70 days pigment formation produced by the very small number of colonies had been very well completed by 24 hours, and almost no difference could be noted when compared with ordinary colonies.

<u>Bacillus prodigiosus subspecies</u>: Colonies subjected to low temperature lessened drastically in growth up to the 7th day (about one-tenth of the original) but produced the same number of colonies. One-seventh of the original still survived even after 70 days. Those in the control media exposed to room temperature had a higher resistance capacity. Although the colony number gradually decreased within 28 days to some extent, the overall number still far exceeded that exhibited by those exposed to low temperature. Only after 49 days was it noted that the number of produced colonies was less than those produced in the low temperature exposure plates. This was again due to the fact that the culture media became dry. Nevertheless, it seemed that they were still able to maintain their vitality. Actually a small number of colonies were found on the culture plate.

In short, bacillus coloni subjected to low temperature exposure such as minus 30 degrees Centigrade, in culture media was not affected to any great extent as far as its colony producing capacity was concerned. But most of the bacillus mycoides and bacillus proteus vulgaris died at low temperature within 7 days and the colony number on the culture plate drastically decreased, although it has been proven that some survived unchanged after 7 days, and some few survived after 28 days.

There was a tendency to gradually decrease as well about the 20th to the 35th day in the colony count on the cultured plate subjected to room temperature, but it was shown that there were more colonies than those exhibited by low-temperature exposure cultures. After the above stated days, they decreased almost to the same degree as those in the experimental subject one point; that is, it seemed that they became, to some extent, fewer in number than those in the experimental group. The reason for this is probably attributable to the fact that the surface of the culture media subject to low temperature for more than 20 to 25 days increased proportively dried; bacteria on the surface of culture media lose their moisture and firmly adhere to the media. As a result, it becomes difficult to take leaphein or colonies and the ability of the bacteria to proliferate is very much weakened.

TABLE 1

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EXPERIMENT NO 1

Experiment Conducted in the Low Temperature Laboratory (ground floor 20 degrees Centigrade) in which Controls were Placed Without Being removed from the Culture Media.*

	Bacillus anthracis		Bacillus mycoides		Bacillus megaterium vulgatus	
	Low Temperature	Control	Low Temperature	Control	Low Temperature	Control
1 Day	2550	[illegible]	[illegible]	[illegible]	2576	[illegible]
2 Days	1580	1290	2722	[illegible]	7725	1755
4 Days	1550	1230	1469	5450	7657	[illegible]
7 Days	1515	1230	1272	5055	896	[illegible]
14 Days	1412	1200	368	2500	836	4618
21 Days	1302	1290	352	2525	722	4567
28 Days	1150	564	300	1545	702	3847
35 Days	1179	477	239	144	720	1200
42 Days	1210	360	231	123	750	1015
49 Days	1160	285	68	50	747	113
56 Days	1050	71	53	49	620	89
70 Days	1050	21	40	10	500	47

[NOTE:

* The figures in this table may not be accurate because the photostat used in the translation was blurred.]

FIGURE NO. 1

EXPERIMENTAL CURVE FOR BACILLUS COLI

Experiment Conducted in the Low Temperature Laboratory in which Bacteria Were Placed Without Being Removed From the Culture Media.

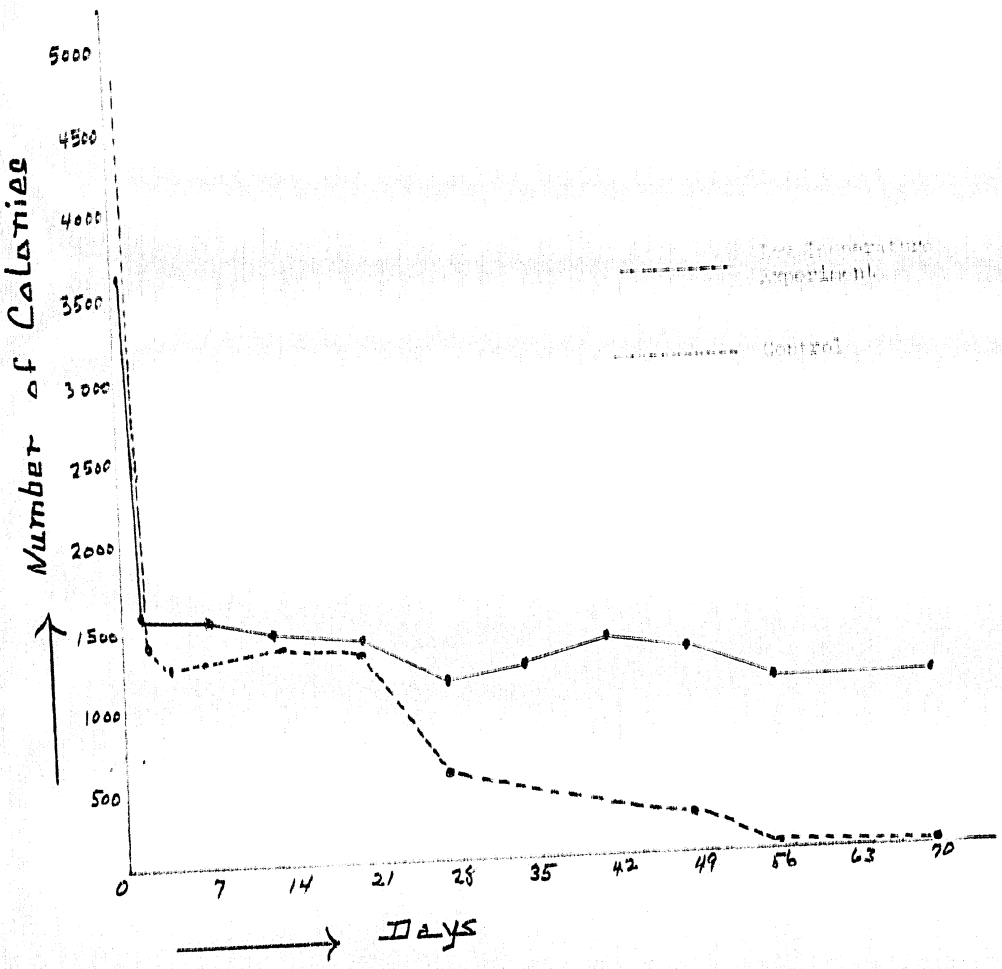
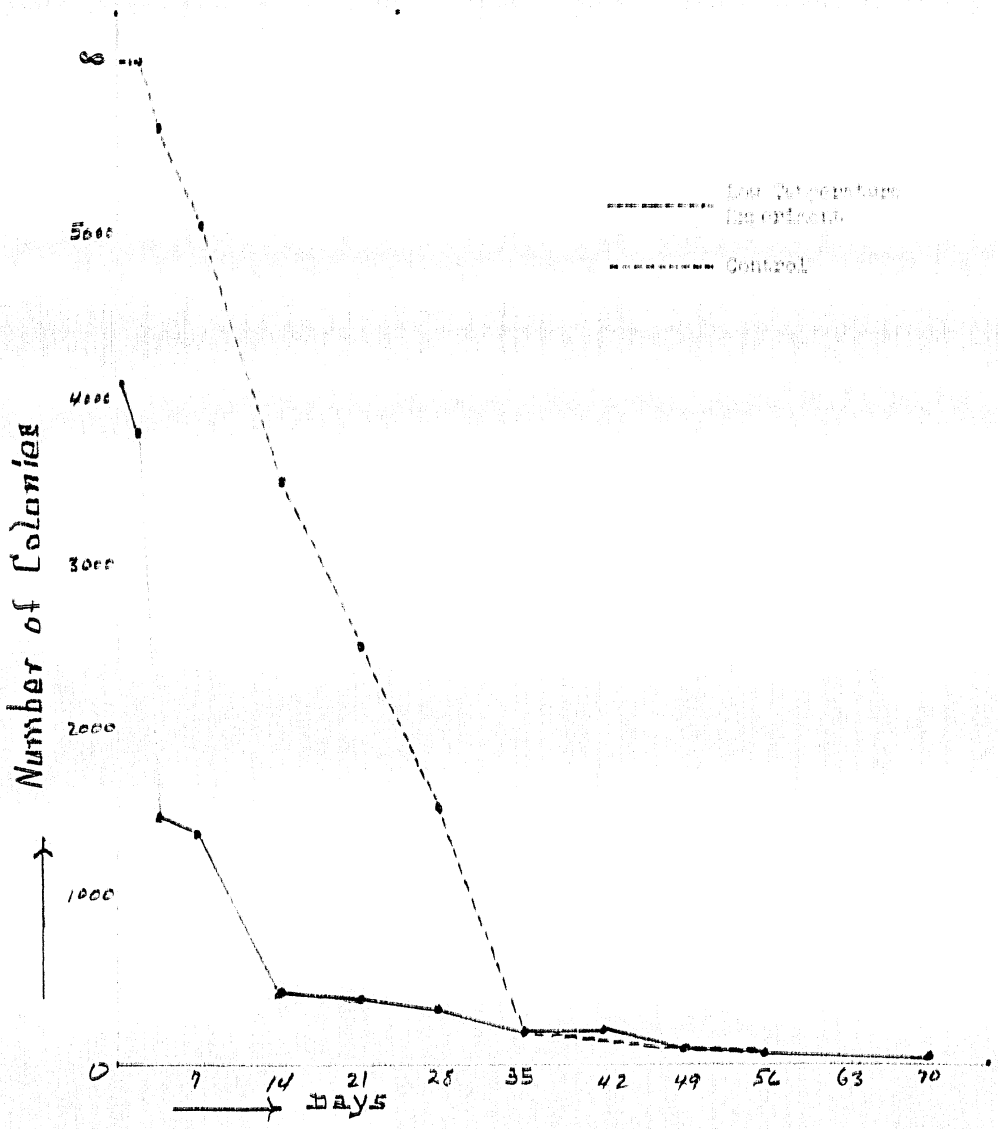


CHART NO. 2

EXPERIMENTAL CURVE FOR BACILLUS PROVIDENCIE

Experiment Conducted in the Low Temperature Laboratory in which Bacteria Were Cultured Without Being Removed From the Culture Media.



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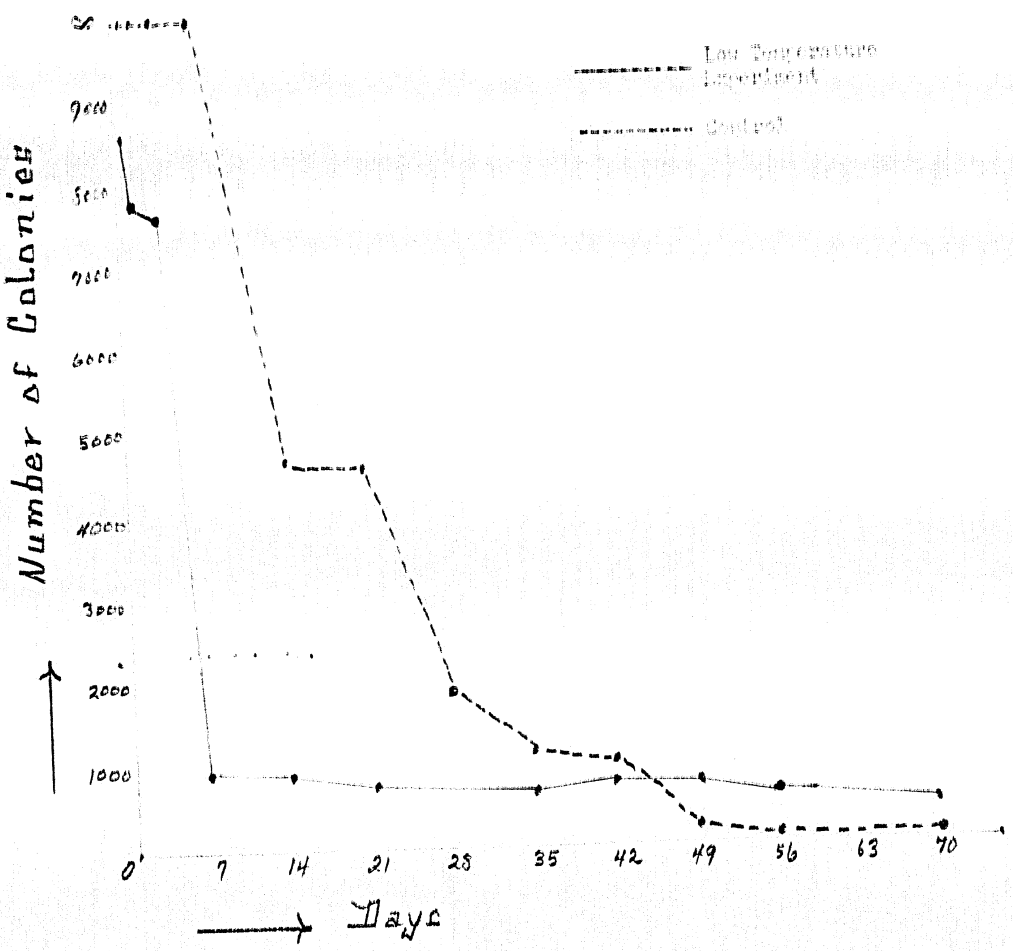
GRAPH NO. 3

EXPERIMENTAL STUDY FOR BACTERIAL POWDER ASSAYS

Experiment Conducted in the Low Temperature Laboratory in which Bacteria Were Placed Without Solid Removed from the Culture Media.



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Experiment No 2

The results of the experiment involving Bacillus coli, Bacillus pyocyaneus and Bacillus proteus vulgaris in a suspension of sterile saline solution, in the low temperature laboratory (around minus 30 degrees Centigrade at normal room temperature are as follows:

Bacillus coli: The colony number produced decreased drastically after twelve hours at low temperature, but decreased accelerratively to seven-tenths of the original number on the 7th day, to one-half on the 14th day, one-sixth on the 21st day, and one-thirtieth on the 28th day. Although they continued to decrease somewhat, they never seemed to die out even by the 105th day.

In the case of those at room temperature, about the same colony numbers were abundantly every day, when the culture media surface did not dry even after many days as in previous experiments. The number of grown colonies decreased only slightly after 94 days.

Bacillus pyocyaneus: The resistance to low temperature was very weak. The colony number grown decreased drastically after only 12 hours, and decreased to less than several percent by the 7th day. After that, a small but similar colony number was produced until the 70th day, but they died after the 84th day.

Bacillus proteus vulgaris: Most of them died after 12 hours as did Bacillus pyocyaneus. The colony number grown decreased to one-sixth on the 7th day after being at low temperature for 12 hours. Then, the colony number decreased slightly each successive day until the 70th day, but by the 84th day all had died. On the contrary, even after 94 days, those at room temperature produced as many colonies as they first had at low temperature, and only after 105 days did they start to produce less.

In short, when these bacteria, as a suspension in sterile saline solution, were exposed to normal room temperature, the colony number remained constant for many days, but decreased somewhat after 84 to 105 days. Those frozen at low temperature showed marked decreases after 12 hours, and the colony number continued to decrease gradually. Nevertheless, they did not completely die out for a long time. Bacillus pyocyaneus and Bacillus proteus vulgaris died within 70 to 84 days, but Bacillus coli calcolaco its vitality over after 105 days.

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TABLE NO 2

EXPERIMENT NO 2

Low Temperature Experiment with Sterile Saline Emulsion.

Incubation Before the Experiment	Bacillus coli		Bacillus cereus		Bacillus proteus vulgaris	
	Low Temperature	Control	Low Temperature	Control	Low Temperature	Control
	Lang	Lang	Lang	Lang	Lang	Lang
12 Hours	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
24 Hours	[illegible]	[illegible]	232	[illegible]	1106	[illegible]
2 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
7 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
14 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
21 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
28 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
35 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
49 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
70 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
84 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
105 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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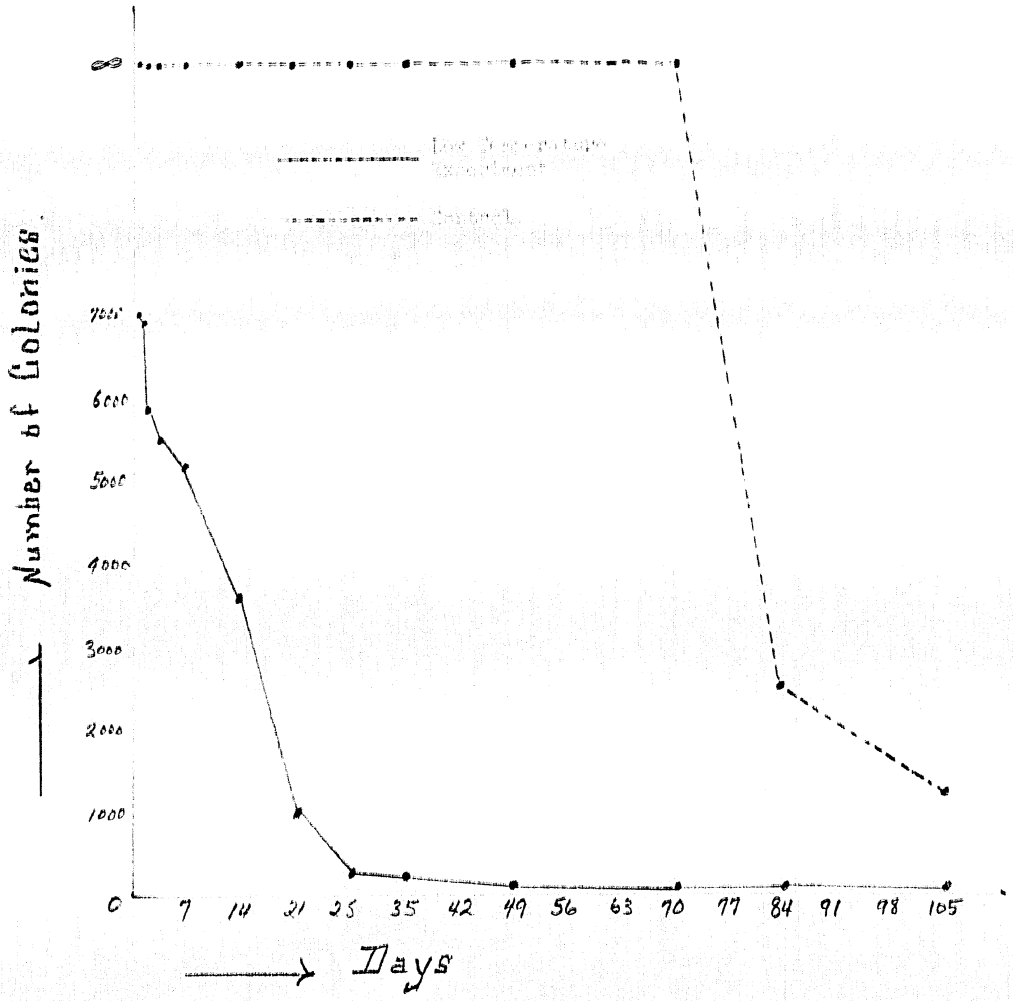
CHART NO 1

EXPERIMENTAL CHART FOR [illegible]

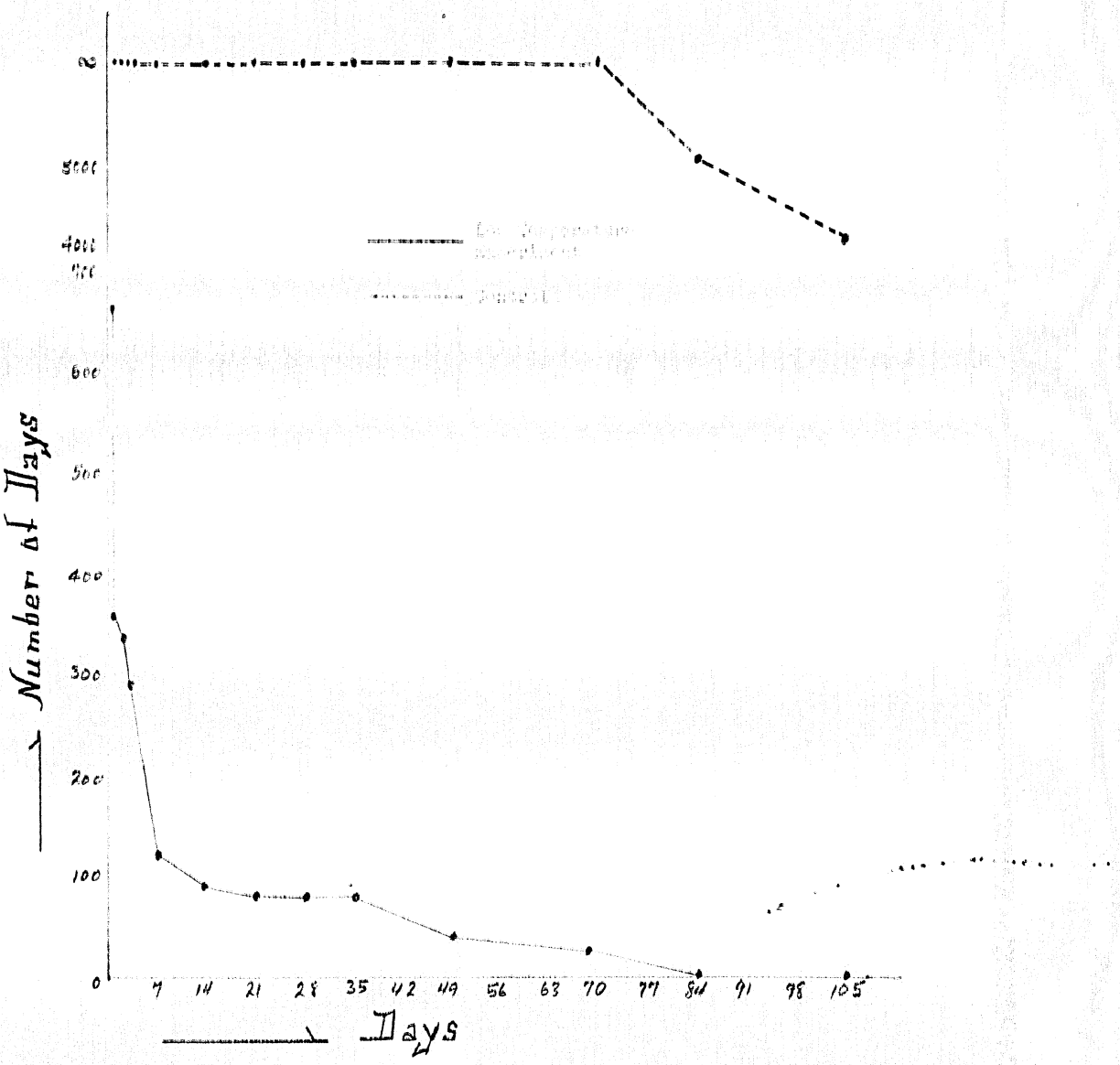
for Temperature Incubator with Sterile Saline Emulsion.

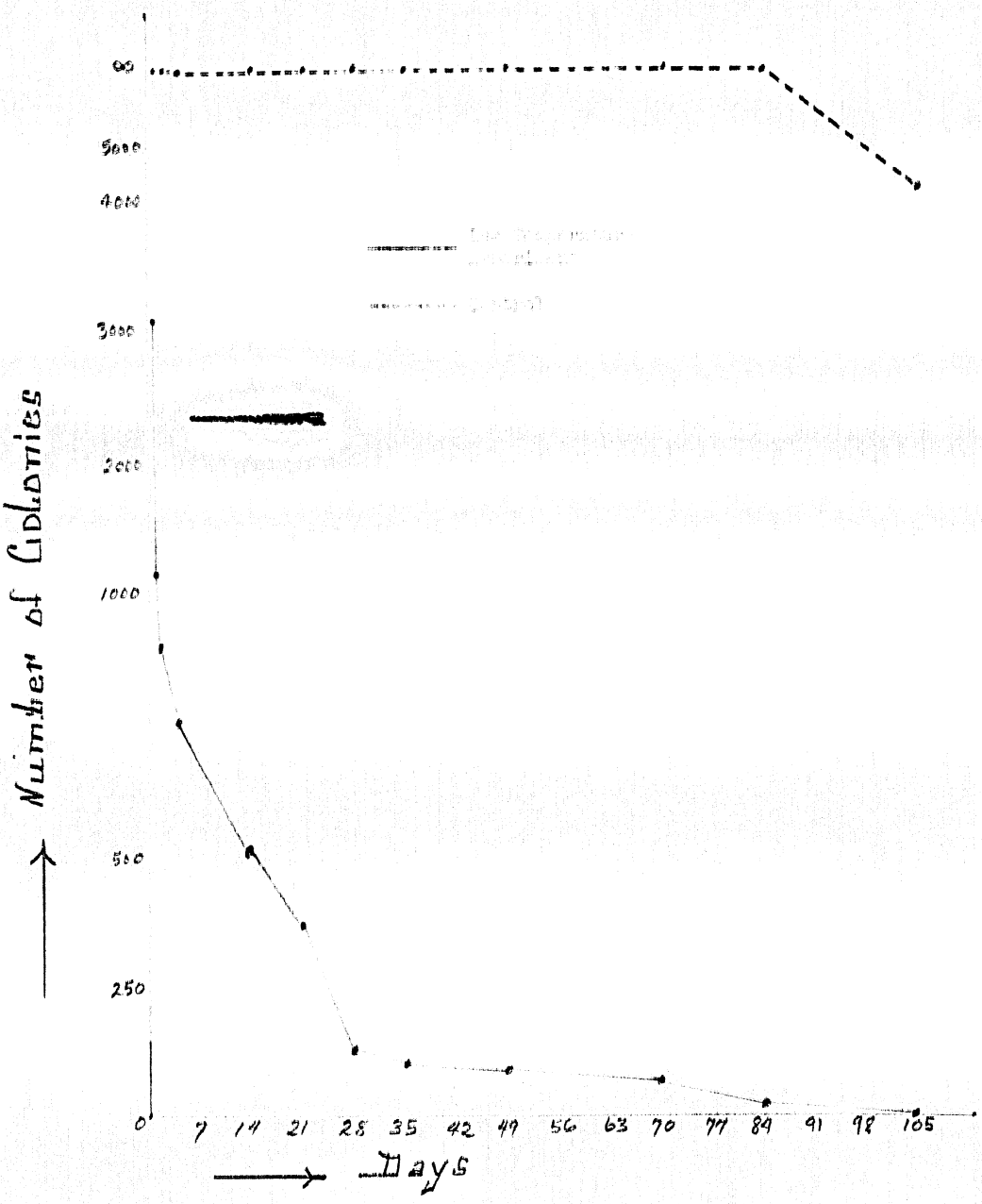


APPENDIX

EXPERIMENTAL CHART FOR BACTERIA PROGRAM

Low Temperature Incubation with Sample Collection.





Summary of Item 1

The resistance of Bacillus colon to low temperature is very strong. In these experiments the bacteria did not die out even after being continuously exposed to a low temperature of about minus 30 degrees Centigrade for a long time. In the case of those cultured at a low temperature for 70 days, about the same number of colonies grew as in those exposed for only 7 days. In saline solution emulsions, the colony number decreased gradually after only 12 hours, and most of them had died by the 28th day. Although the number decreased, the reduction subjected this vitality even after 105 days.

The resistance of Bacillus pyocyaneus and Bacillus proteus variants to low temperature is far weaker than that exhibited by Bacillus coli. In the saline solution emulsions, the colony number decreased considerably after 12 hours. Between the 7th and 14th day, only one-sixth of the number present after 12 hours at low temperature remained. Thereafter, small numbers of colonies were not all died out between 21 and 105 days. In the case of those grown on the culture media, the colony number decreased to a very great extent within a few days after exposure to low temperature, but a few survived and produced colonies even after 70 days.

In the case when cotton placed culture media was used in exposing the bacteria to simple room temperature as contrasted with those exposed to low temperature, the death rate was greatly reduced in the shorter period of 7 to 28 days, and many colonies were produced. But after 28 to 35 days, the bacteria's vitality weakened as the culture media died. Even when applied to the culture media surface, the colony number decreased sharply to almost the amount found at low temperature. There was a tendency for the colony number on control plates to decrease.

On the other hand, since the saline solution emulsion does not dry, bacteria on these control plates lived and maintained their vitality for a relatively long period. Consequently, the latter experiments better illustrated the results of bacterial resistance.

Item 2. The Effect of Low Temperature On Bacillus Typhosus

The resistance of Bacillus typhosus varies depending on its solvents, life span, and other varying conditions. It was stated that Bacillus typhosus lived for 2 months or more in tap water and from 3 to 5 weeks in sterile water. The material related to the experiments on the resistance of Bacillus typhosus to low temperature was studied in the literature. It was noted that Fruitgen (1887) proved that Bacillus typhosus in ice at outdoor temperatures of minus 1 degree Centigrade to minus 21 degree Centigrade survived for 2 months, but disappeared 2 days when dissolved and then refrozen 5 times. On the other hand, Lentefuscc (1893) differed with this theory as he believed that Bacillus typhosus does not die, but maintains its vitality even after being alternately exposed to the low temperature of minus 15 degrees Centigrade and the incubator temperature of 37 degrees Centigrade. The only result was the termination of its propagation function. It stated further that Bacillus typhosus mixed in water or stool did not lose its virulence even when exposed to low temperatures of minus 10 degrees Centigrade to minus 15 degrees Centigrade for up to 6 hours. Janowski (1890) stated that it was possible for Bacillus typhosus to survive cold exposure for a long time under normal conditions. Brehme (1901) proved that some Bacilli typhosus in a bouillon culture media still survived at minus 2 degrees Centigrade to minus 16 degrees Centigrade after 140 days, and even after being alternately exposed to plus 15 degrees Centigrade and minus 15 degrees Centigrade 40 times within 36 hours.

Reverel (1900) proved that Bacillus typhosus, Bacillus anthracis, and Bacillus diphtherise survived after being exposed to liquid air for an hour.

Cedrick and Winslow (1900) found that 30 to 40 percent of the cultured Bacillus typhosus died within 1 hour after being exposed to freezing temperature, that only 1 percent survived after 2 weeks, and a very few colonies persisted even after 12 weeks. He also stated that the resistance of bacteria varied greatly according to the species.

Park stated that Bacillus typhosus survived even after being exposed to low temperature for 12 weeks but died after 22 weeks. He further stated that Bacillus typhosus maintained their vital functions even after being exposed to liquid air for 120 minutes.

Levy and Mayer (1903) tried their experiment under completely natural conditions and proved the existence of Bacillus typhosus in a typhoid patient's stool when they preserved for 5 months, the first check being made in September and the second one in February. He later proved that Bacillus typhosus from stools remained potent for 15 days (from 6 February to 20 February 1903) when used as fertilizer on cultivated lands with the temperature ranging from 11.6 degrees Centigrade to minus 5.4 degrees Centigrade.

Page and Stephan conducted low temperature experimentations with Bacillus colon and Bacillus typhosus and arrived at the following conclusions:

(1) 90 to 95 percent of both types of bacteria died after being put into water at 23 degrees Centigrade for 24 hours, but a few colonies were able to survive for a long time;

(2) 50 percent of the cultured Bacillus colon and 75 percent of

Bacillus typhosus died in 15 minutes when placed in ice, 95 percent of the Bacillus colon and 98 percent of Bacillus typhosus died in 1 hour, and 99 percent of both bacteria died after 24 hours. Only a few colonies survived under this freezing condition for a long time. Bacillus colon survived for 3 months and Bacillus typhosus for 9 months.

Tezzi (1903) prepared Bacillus cholerae, "domestic fowl" Bacillus cholerae, Bacillus diphtheriae, Bacillus mallei and Bacillus typhosus in bacterial water suspensions. He froze them at minus 21 degrees Centigrade and melted them in a water bath tank maintained at 37 degrees Centigrade for 12 thirty-minute intervals. They were then cultured on bouillon plates and after 18 hours melted for the last time. He then found no change in the bacteria's morphology or virulence. He found only slower development and slight disturbances.

Chafalon (1904) reported that he put Bacillus typhosus in liquid air at minus 180 degrees Centigrade for 6 months and found that it still possessed its pathogenicity and its positive agglutination reactions.

Perrone (1907) stated that animal serum injected with Bacillus typhosus frozen for 12 hours at minus 15 to minus 17 degrees Centigrade showed strong agglutination reactions. Also, the development of Bacillus typhosus frozen at the same temperature and for the same length of time was greatly weakened, but the pathogenicity was regained if returned to room temperature in about 12 hours after removal from the freezing conditions.

We will consider now the research results obtained in this country on the influence of winter low temperature on Bacillus typhosus. Otsuka stated that when he was stationed with the army in the winter of 1905, he soaked Bacillus typhosus onto sterile cotton thread. After

incubator-drying, they were put in a Petri dish, exposed to light and then put in the shade for 10 hours to 1 week. They were then removed and cultured. He found that the bacteria still survived though he made his observations for only 1 week.

Then, Katsutani and Kasuga studied, in 1920, the growth of Bacillus paratyphosus A by putting them in a bouillon culture for 16 hours. They then froze them for a specific time at minus 30 degrees Centigrade and then cultured a loopful in ordinary agar culture media. He found that those exposed to cold conditions for 10 days died without producing a single colony. He observed also that those seeds of bacteria exposed to the cold climate for a short time during several successive generations increased their capacity to resist low temperatures.

Kusa (1929) studied the resistance exhibited by Bacillus typhosus and Bacillus paratyphosus B. He stated that bacteria mixed with ordinary peptone water and then exposed to temperatures ranging from plus 5 degrees Centigrade to minus 15 degrees Centigrade for 60 days. He all find growth somewhat retarded. Once put in incubators at the proper temperature for 72 to 96 hours, the bacteria recovered their vitality. He stated also that the degree of pigmentation and the agglutination reaction of bacteria were not influenced by the above process, though the bacteria became inactive.

Kiku also studied the resistance of Bacillus typhosus, Bacillus dysenteriae, Bacillus coli, and Bacillus cholerae when exposed for a long time to outdoor temperature during the winter of 1927-1928 in Dalian. He observed Bacillus typhosus subject to outdoor temperatures ranging from minus 20 degrees Centigrade to 7 degrees Centigrade for 45 days. Those mixed with stool and urine died in 30 to 40 days, but those mixed

with tap water lived 45 days. On the other hand, bacteria in the agar slant died within 20 days, much before those in water, urine, and stool died. The vitality of those alternately exposed to low temperature and room temperature (plus 16 to 25.4 degrees Centigrade) did not differ with those exposed to the low outdoor temperature. Bacteria attached to a silk thread and exposed to outdoor temperature died before those lived with tap water, urine, or stool. They died within 30 to 40 days.

Matsumoto (1930) experimented and proved during the cold season (ranging from plus 12 to about 24 degrees Centigrade) in the Manchuria District that Bacillus typhosus cultured in an ordinary agar slant for 4 hours still possessed the ability to grow even after 64 days. He showed that they maintained their vitality for at least 77 days on fresh culture media.

Watanabe and others (1937) studied the resistance of Bacillus typhosus in stool and urine. He proved that they died in 10 days during the mid-summer and in about one and a half months to 2 months during the spring and autumn, while in the winter they survived for almost 6 months if they were preserved at temperatures below the freezing point.

In conclusion, there are uncontrolled temperature variations from day to day and inconveniences arose from this inability to establish a specific temperature at any fixed time. This was the natural result from using outdoor air temperatures during the above stated various experiments. Yet, it has been proven that Bacillus typhosus can maintain its vitality under low temperature over a prolonged period.

The results of my own low temperature experiments are shown in Table 3.

Experiment No 1

I exposed Bacillus typhosus and Bacillus paratyphosus A and B on an agar slant to approximately minus 30 degrees Centigrade. I then counted the colony numbers in one loopful with similar means as previously described. There was no marked change in the group colony number of Bacillus typhosus up to the 21st day. Twenty-eight days later, the colony number decreased to about one-third of the original, and even after 70 days approximately one-seventh of the original number still possessed vitality.

On the contrary, due to the dryness of the culture medium surface at room temperature, most of the bacteria in the control media died after 14 days while at the same time the colony number decreased to one-twentieth to one-hundredth, far less than the percentage at low temperature. Although not many differences were evident up to the 7th day as compared with those at low temperature, only a small fraction of them survived after 70 days.

There was not much difference in the death rate of Bacillus paratyphosus A at low temperature and at room temperature. On the 14th day, the colony number decreased drastically to one-fifth of the original number. After that, they decreased gradually up to 50 to 70 days when the colony count decreased drastically to one-twentieth of the original number. These remaining bacteria still maintained their vitality. That is to say, this bacteria's resistance is similar to that of Bacillus typhosus, and the colony number grown in the room temperature control was maintained rather well for a long period.

The resistance of Bacillus paratyphosus B to low temperature was very great. Up to approximately 49 days, the colony number was almost

similar to the original number. Although they decreased to one-third of the original number between the 49th and 70th day, the colony number was far more than those grown at room temperature. The colony number at room temperature decreased drastically as compared to those exposed to low temperature. The decrease accelerated progressively each day, to one half by the 7th day and one-tenth by the 14th day. The decrease became relatively slower after 14 days, but only one-one-hundredth of the original number developed but survived after a 70 day period.

In summary, the resistance of Bacillus paratyphosus A to low temperature is less than Bacillus typhosus. On the 7th day, the former decreased to one-fourth of the original colony number, while the latter maintained approximately the same number up to about 21 days, although later decreased to one-fourth after 28 days. The resistance of Bacillus paratyphosus A is much greater, on the other hand. The green colony number was similar to the original until about the 14th day. Then, it decreased gradually. Even after 70 days, a few colonies survived.

All 3 bacteria in the control media decreased to one-fifth or one-tenth of the original number, due to the culture medium dryness, in a 7 to 14 day period. Thence, as in the experiment made at low temperature, a small but approximately similar colony number grew until the 70th day, although the other 2 bacteria, other than Bacillus paratyphosus A, produced somewhat fewer colonies than at low temperature. I believe this is a good experiment illustrating the worse effects of dryness on bacteria as compared to the effects of low temperature.

TABLE No 2

SUPPLEMENT NO 1

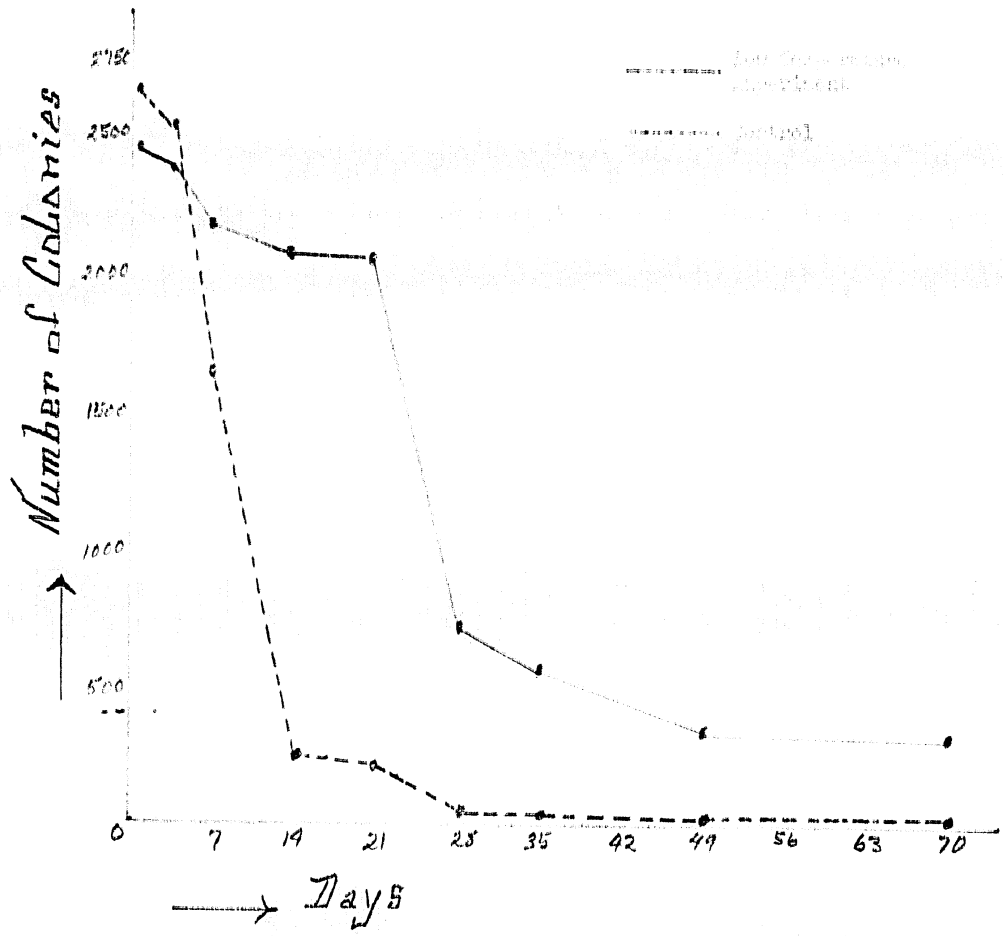
Experiment in the Low Temperature Laboratory (around minus 30 de-grees Centigrade) in which protein Meat Placed Without Seal [illegible] [illegible] Bacteria Growth.

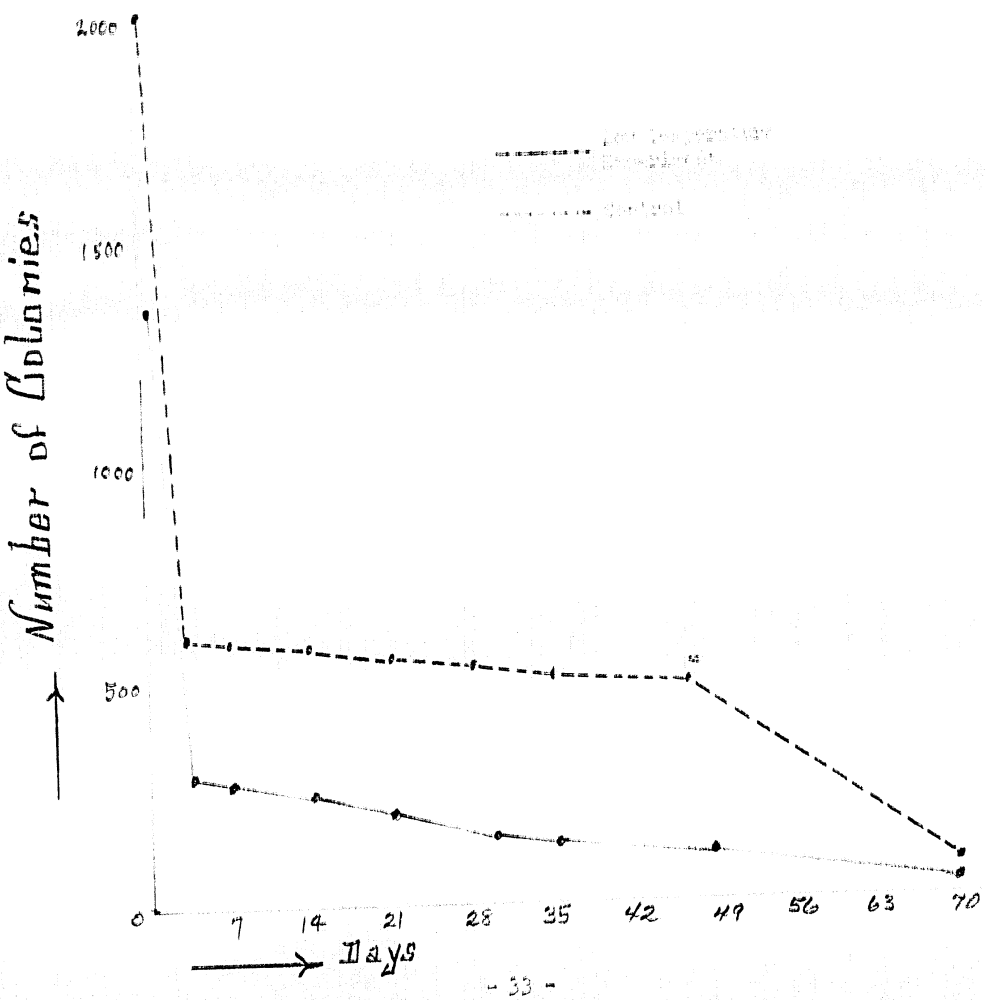
Sealing Methods	Sealing Methods A	[illegible]
Low	Low	Low
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

[illegible]

[illegible] Growth of Bacillus Cereus

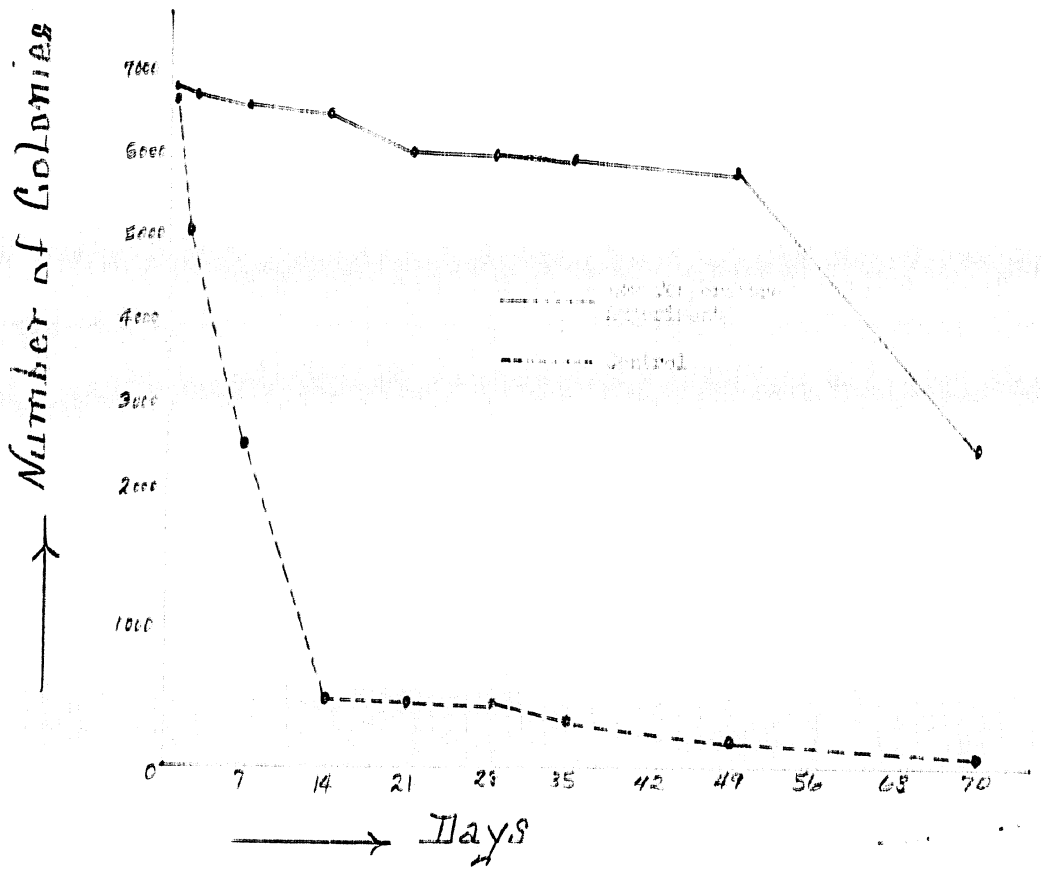
Experiment in the Low Temperature Laboratory in which Controls [illegible] Were Without Sodium Lauryl Sulfate in the Culture Media.





[illegible]

Experiment in the Low Temperature Laboratory, Arctic Lab Detachment [illegible] placed [illegible] removed upon the outdoor yards.



Experiment No. 2

Bacillus typhosus and Bacillus paratyphosus A and B were suspended in a sterile saline solution by similar means as previously stated and then exposed and frozen at low temperature. The results are indicated in Table 2.

The growth of Bacillus typhosus colonies on the experiment's first day was relatively slower as compared with the control. On the fourth day, the colony number equalled three-fourths of the original number. On the 18th day, only one-fourth remained and only one-eighth by the 21st day. After that similar colony numbers were produced until the 49th day when each of the colonies died and no new colonies were produced. The colony number in the control bacterial suspension subjected to room temperature decreased gradually but always equalled more than those exposed to low temperature. Even after 105 days there were as many colonies as had been present in the low temperature experiment plates on the 1st day.

The case of Bacillus paratyphosus A was similar to that of Bacillus typhosus. On the 1st day of the experiment, the colony number decreased drastically as compared with the original amount and was far less than present in the control. Between the 4th and 18th day, they decreased to half of those present on the first day. By the 21st day they had decreased to one-eighth of the original number and to one-fifteenth by the 70th day, although some were still living in small numbers even after the 105th day. On the contrary, the bacterial suspension exposed to room temperature produced tremendous numbers of colonies which were almost incalculable up to the 49th day. On the 70th day, the number decreased drastically to one-third of that on the 49th day, but few changes occurred after that and colonies were produced in greater quantity than that exposed to low temperature.

The suspensions of Bacillus paratyphosus B at room temperature produced many colonies without being affected in any way even after 105 days. But those at low temperature produced less than those in the control only 12 hours later, and the colony number on the 24th hour was one-half of those present on the 12th hour. They decreased to one-third or one-fourth between the 4th and 7th day. Then the colony number decreased gradually until the 105th day. Then the number equalled one-tenth of compared with the amount present when the experiment started.

In brevity, Bacillus typhosus and Bacillus paratyphosus A and B in sterile saline solutions were affected considerably during the first 12 days when preserved at the low temperature of about 20 degrees Centigrade. Although, in each case, the colony number decreased considerably, they did not die out completely, for a small number survived. The reactions exhibited by Bacillus paratyphosus B and the dysenteric and Bacillus typhosus the weakest. Bacillus paratyphosus A and B still partially survived even after 105 days, Bacillus typhosus succumbed to their vitality within 84 days.

TABLE NO 1

EXPERIMENT NO 9

Low Temperature Experiment with Sterile Saline Emulsion.

	Bacillus typhosus		Bacillus paratyphosus A		Bacillus paratyphosus B	
	Low		Low		Low	
Hours after the injection	Temperature	Control	Temperature	Control	Temperature	Control
	Log	Log	Log	Log	Log	Log
30 Hours	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
1 Day	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
7 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
14 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
21 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
28 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
35 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
49 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
70 Days	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
84 Days	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
105 Days	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 10.10

Bacterial Counts for Various Methods

Low Temperature Experiment with Specific Saline Isolator.

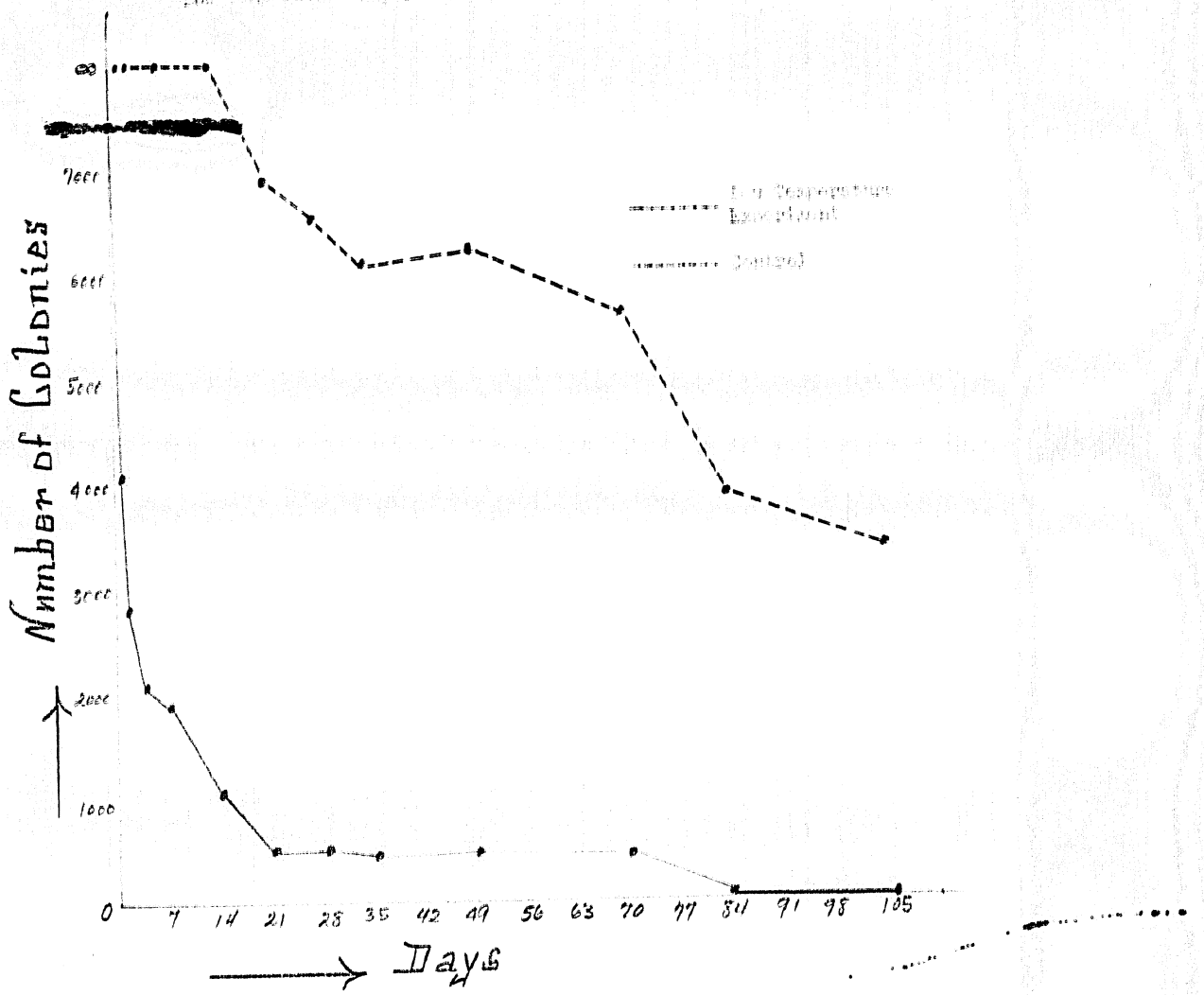


Chart 10-11

Graphical Chart for Bacillus Malthusus A

Low Temperature Experiment with Sterile Saline Washes.

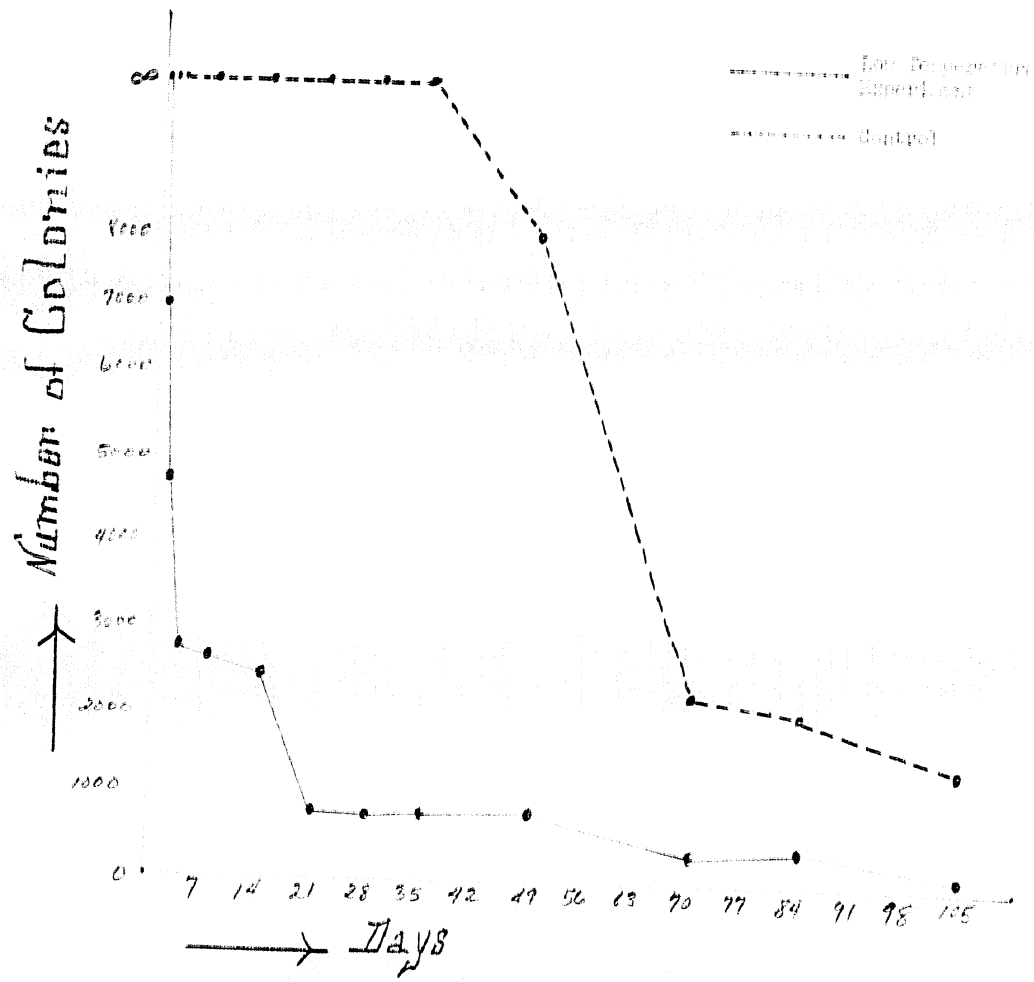
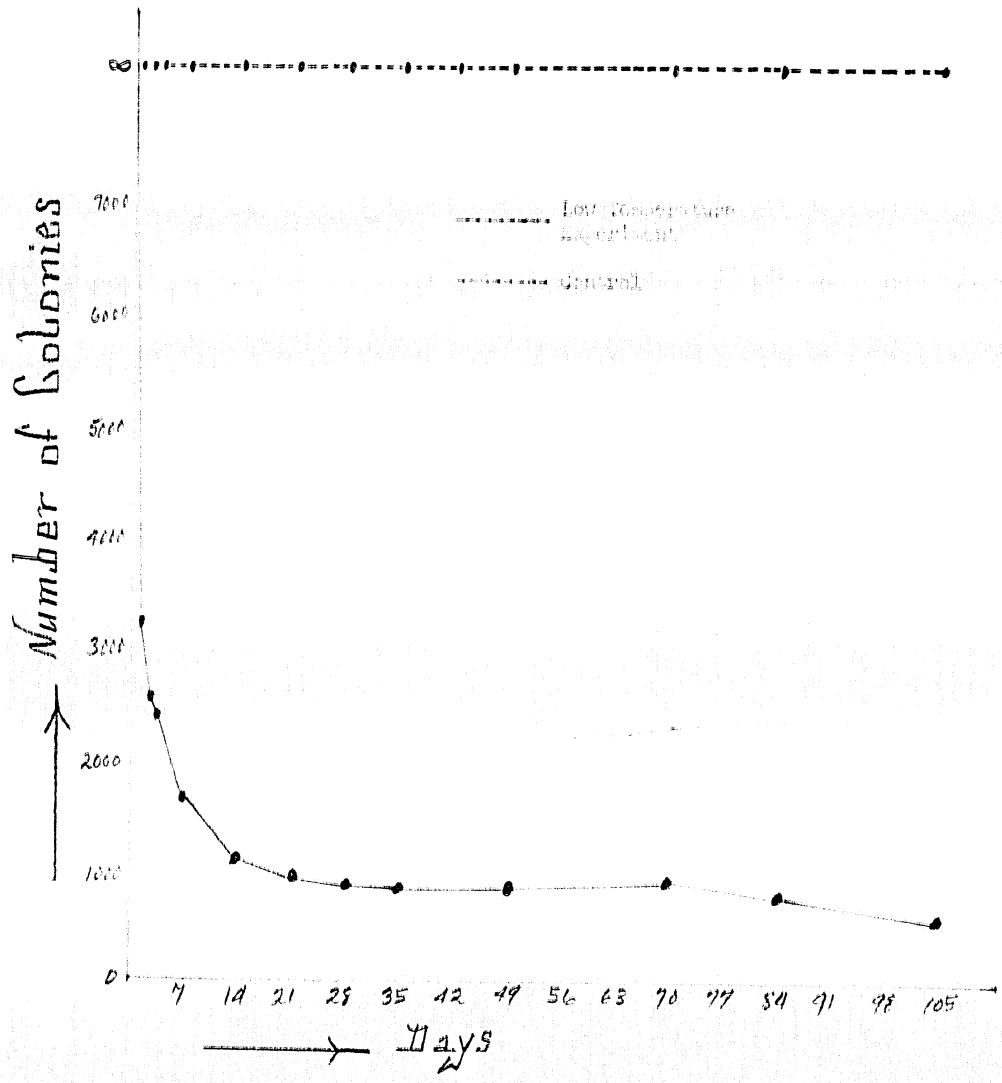


CHART NO. 11

EXPERIMENTAL CHART FOR BACILLUS PARATYPHOSUS B

Low Temperature Experiment with Sterile Saline Emulsion.



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Summary of Item 1

In the low temperature laboratory experiment where the 3 above stated bacteria in the culture media were exposed the results were almost similar. That is, the colony number decreased each day, but some remained even after 70 days. On the contrary, in the case of the control exposed to room temperature, the culture media dried and the colony number lessened accordingly. The development power in that case increased and weakened by the 70th day as compared with those at low temperature, although a portion still survived.

According to the above stated results, which were similar to the previous experiments, the influence of the culture media dryness on the living capacity of bacteria was far more than the influence of the low temperature of minus 30 degrees Centigrade. In the case of the alcoholic saline suspensions, the control media did not dry, and the colony number did not change much despite the low element. On the contrary, the vitality of those at low temperature was weaker than those in the control. In this case, Bacillus typhosus died completely in 84 days, while the other 2 bacteria survived even after 105 days. Thus, we know that the resistance of the above 3 bacteria to low temperature, such as minus 30 degrees Centigrade, was rather strong.

Thus, it is seen that those pathogenic microorganisms exposed to a cold climate can preserve their vitality for a long period if the surrounding media does not become dry.

Item 3. The Effect of Low Temperature on Bacillus Dysenteriae and Bacillus Dysentriae Infantum

Since Bacillus dysentriae does not form spores, its resistance to outside stimuli is not too strong. The life span of this bacteria

differs, according to various experimental workers. In water which was neither sterile nor pure it died in 2 to 6 days (Vincent); in sterile distilled water it died in 10 to 12 days (Vincent); in boiling water in 71 days; and in sterile city water in 72 days (Bodkowski).

It is also said that when this material was attached to a patient's underwear it died in 8 days (Casoulet); when attached to the seat, it died in 2 weeks (Blachman); and when attached to the dry cloth it died after 17 days (Ewald).

Nevertheless, the resistance to low temperature is rather strong. It survived longer than those exposed slightly to room temperature (Vincent). According to Reichel, Bacillus dysentriae survived after being exposed to outdoor temperature for 24 days.

In Japan, Hihara made a study under the outdoor temperature conditions prevailed in Pukuoka in Fukuoka Prefecture 7° to plus 10 degrees Centigrade during the winter of 1927 and 1928. He proved that Bacillus dysentriae obtained from city water died in 30 to 40 days; it lived in stool and urine for 50 to 55 days, but died within 14 days on culture media; it lived 8 days in the city water at room temperature but died within 8 days in stool and urine at the same temperature. In every case, he proved that those subjected to low temperature lived longer than those subjected to room temperature. Moreover, Bacillus dysentriae attached to dry clothes died after 40 to 50 days when exposed to outdoor temperatures, and when exposed every other day to the low temperature of minus 20 to 0 degrees Centigrade. With room temperatures of plus 16 to 25.4 degrees Centigrade, it died somewhat sooner than those exposed to the outdoor temperature.

Kasuga and Matsutani declared that Bacillus dysentriae cultured

in broth for 18 hours died between 10 and 30 days after being exposed to the low temperature of minus 30 degrees Centigrade. Hama declared that the colony number of the Shiga type, when exposed to low temperature, decreased slightly each day, but after 70 days decreased to one-half of the total number present at the beginning of the experiment. This indicated that it possesses a strong capacity to resist low temperature. On the other hand, the control placed at room temperature, despite strict paraffine sealing, continued dying each successive day. In this case, the bacteria lost its vitality and almost completely died out. On the 7th day of the experiment, the colony number dropped to $\frac{1}{3}$ of the original, it dropped to one-seventh on the 14th day, to one-eighteenth on the 28th day, and to only one three-hundredth after 70 days.

Experiment No. 1

Bacillus dysenteriae Shiga A

When this bacteria was exposed to low temperature, the colony number on the 1st day of the experiment equalled only 1/3 of those at room temperature. Later on, the colony number increased gradually, but after 70 days dropped to only $\frac{1}{2}$ of the original number. When the control was exposed to room temperature, the gradual drying caused the colony number on the 7th day to drop to 2/3 of the original amount; it dropped to one-third on the 21st day, to one-sixth on the 35th day, and to one-thirtieth on the 49th day. The colony number of cultures exposed to low temperature became roughly similar to those exposed to room temperature on the 28th day and from then on the former produced far more colonies.

Bacillus dysenteriae Flexner A

The resistance capacity of this organism is very great against low temperature. On the 4th day of the experiment, the bacteria exposed to both low temperature and room temperature decreased to seven-tenths of the original number. Yet, those subjected to low temperature suffered

no effects after the 4th day and produced rather large numbers of colonies even after 70 days. On the other hand, those exposed only to room temperature decreased as the culture media dried. On the 14th day, only one-fifteenth of the original amount remained. This number is far less than the number produced by cultures exposed to low temperature. The number continued to decrease each day according by the 21st day to only one-twelfth of the original total. It dropped to less than one one-hundred-and-sixty-ninth on the 49th day, and only 2 colonies were produced after 70 days. This was considerably less than the amount resulting from the cultures exposed to low temperature.

Bacillus dysenteriae infantum (Shiga-Kiyota Bacillus)

The number of colonies in a culture of this bacteria decreased at the same rate both under low temperature and at room temperature. On the 14th day, it decreased to [illegible] of the total to from one-fifth to one-fourth on the 28th day, and after 70 days, only about one-tenth remained. However, this remainder did not die out entirely in 70 days whether exposed to low temperature or room temperature.

Therefore, it is to be noted that the resistance of the 4 various bacteria to low temperature was rather strong, while that of Bacillus dysenteriae Shiga type and Bacillus dysenteriae Komagome B was especially strong. When Bacillus dysenteriae Shiga type in the culture media was removed from the low temperature of minus 30 degrees Centigrade, almost as many colonies as had been present the first day were produced over a 70 day period. Also, for 70 days, Bacillus dysenteriae Komagome B retained, over the same seventy day period, half of the original colony number. The resistance of Komagome A and Bacillus dysenteriae infantum was slightly weaker, and their colony number decreased gradually.

On the other hand, when they were exposed in the culture media to room temperature, as the media dried the bacteria died quickly. Seventy days later, most of them had died, with only a few still persisting.

Lastly, the resistance to dryness of these 4 bacteria types is weak.

TABLE NO 5

EXPERIMENT NO 1

Experiment in the Low Temperature Laboratory (around minus 30 degrees Centigrade) in which Bacteria Were Placed Without Being Removed From the Culture Media.

	Bacillus dysentriae Shiga Type		Komagome A		Komagome B		Bacillus dysentriae in fantum (Ohara-Hinota)	
	Low Temperature	Control	Low Temperature	Control	Low Temperature	Control	Low Temperature	Control
1 Day	6085	5968	1864	6210	10170	11695	4237	5085
4 Days	6695	2695	1352	5260	6902	5253	2260	3955
7 Days	6582	2464	1332	4520	6729	4802	2125	3051
14 Days	5384	877	1293	2573	6627	856	2092	2516
21 Days	5210	560	1224	2316	6559	480	1985	2373
29 Days	4883	85	1025	1525	6320	400	1412	1073
35 Days	4624	50	1123	1299	6219	180	1073	1085
49 Days	3987	30	985	184	6200	70	1316	1129
70 Days	3826	21	923	127	5085	2	32	565

RESTRICTED

GRAPH 10-13

EXPERIMENTAL DATA FOR BACILLUS STERNUTAR SPORE TYPE

Incubated in the Low Temperature Laboratory in which Bacteria were placed without being removed from the Gaseous Media.

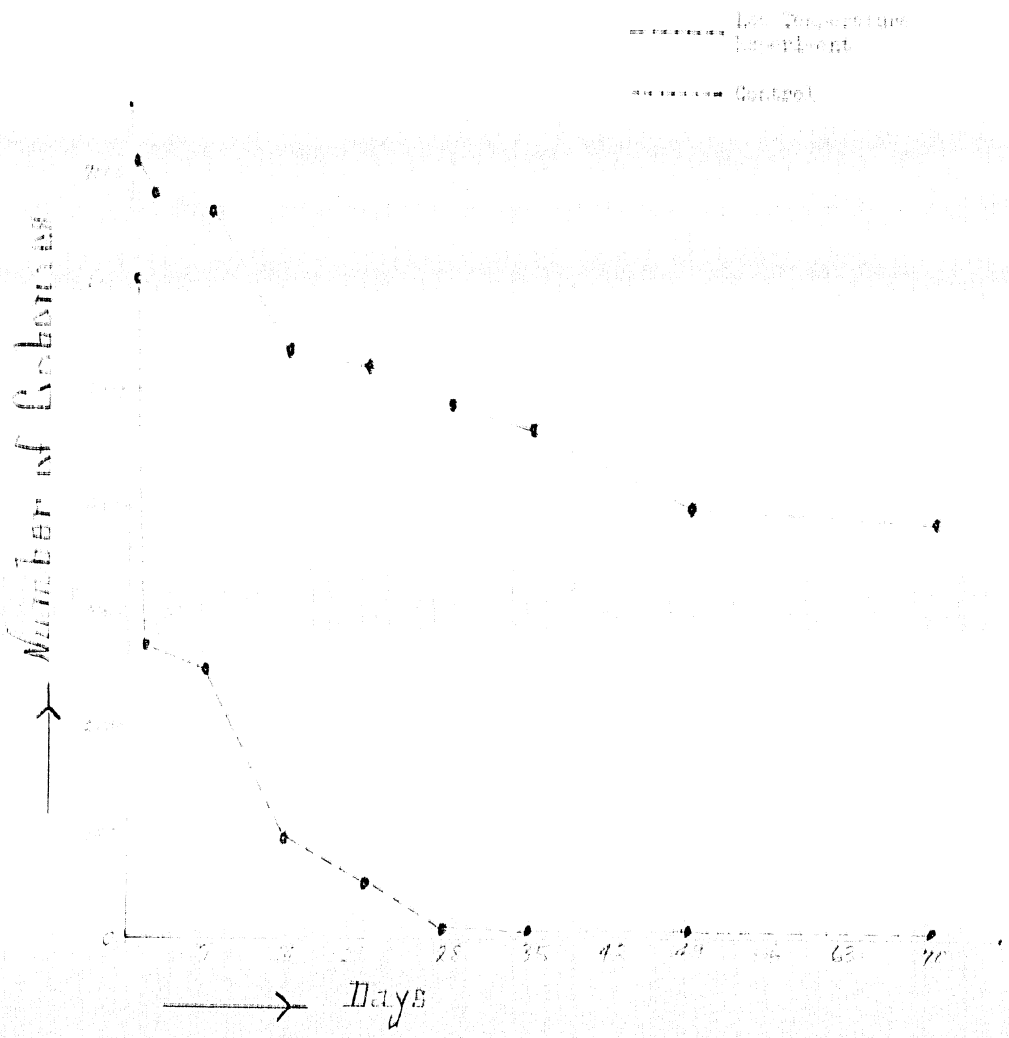

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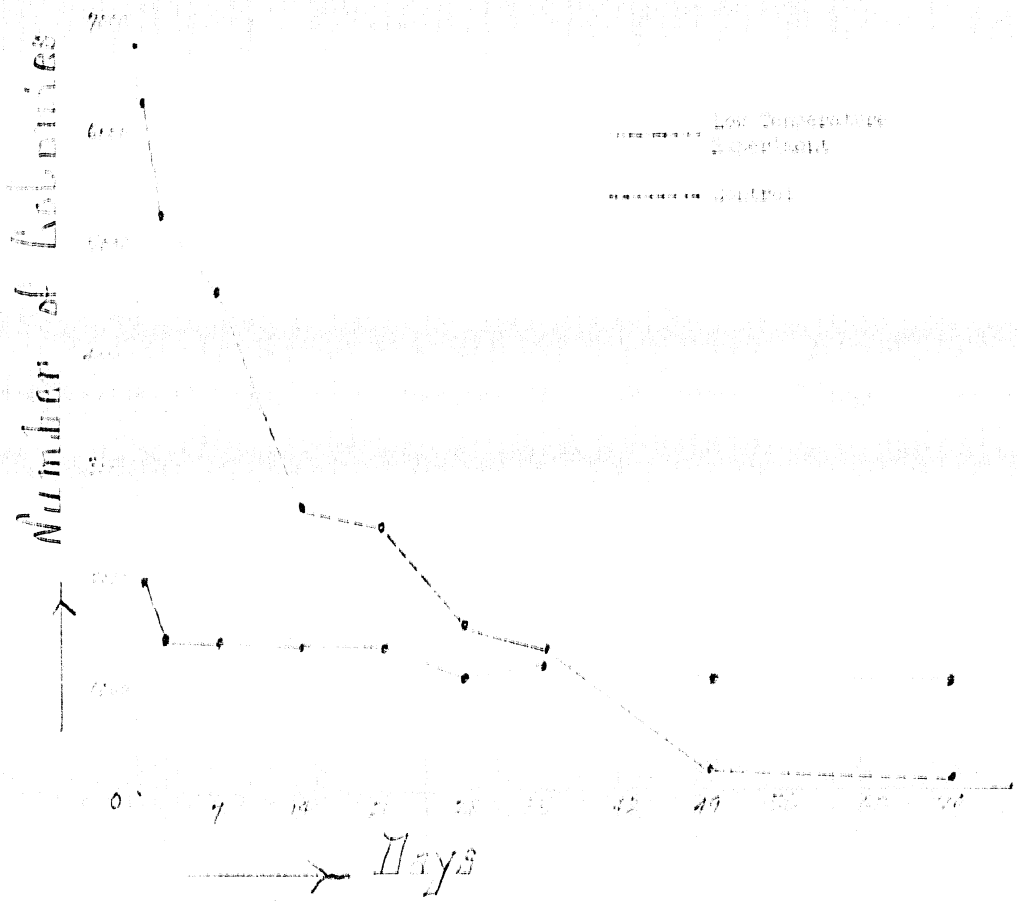
FIGURE NO 14

EXPERIMENTAL CURVE FOR ROCKEFELLA

Experiment in the Low Temperature Laboratory in Which Bacteria Were Placed Without Being Removed From the Culture Bottle.



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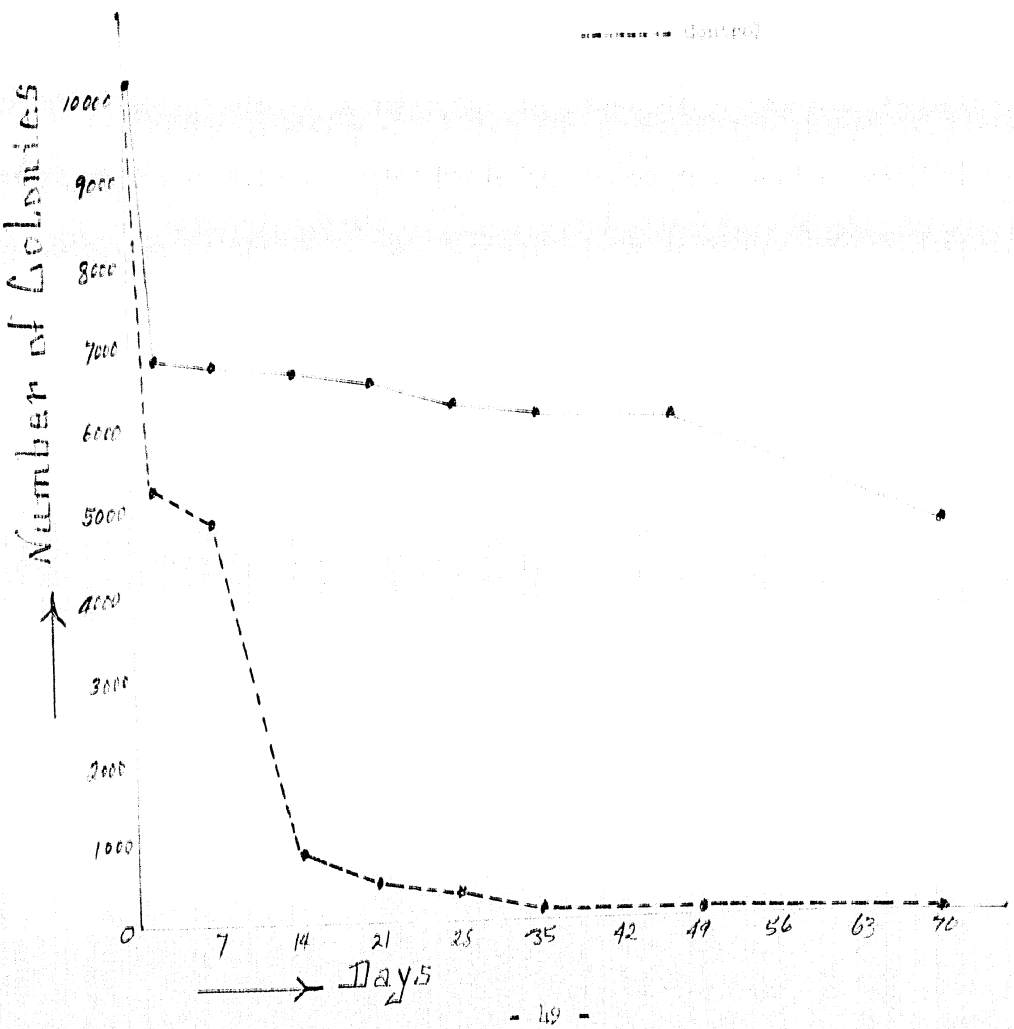

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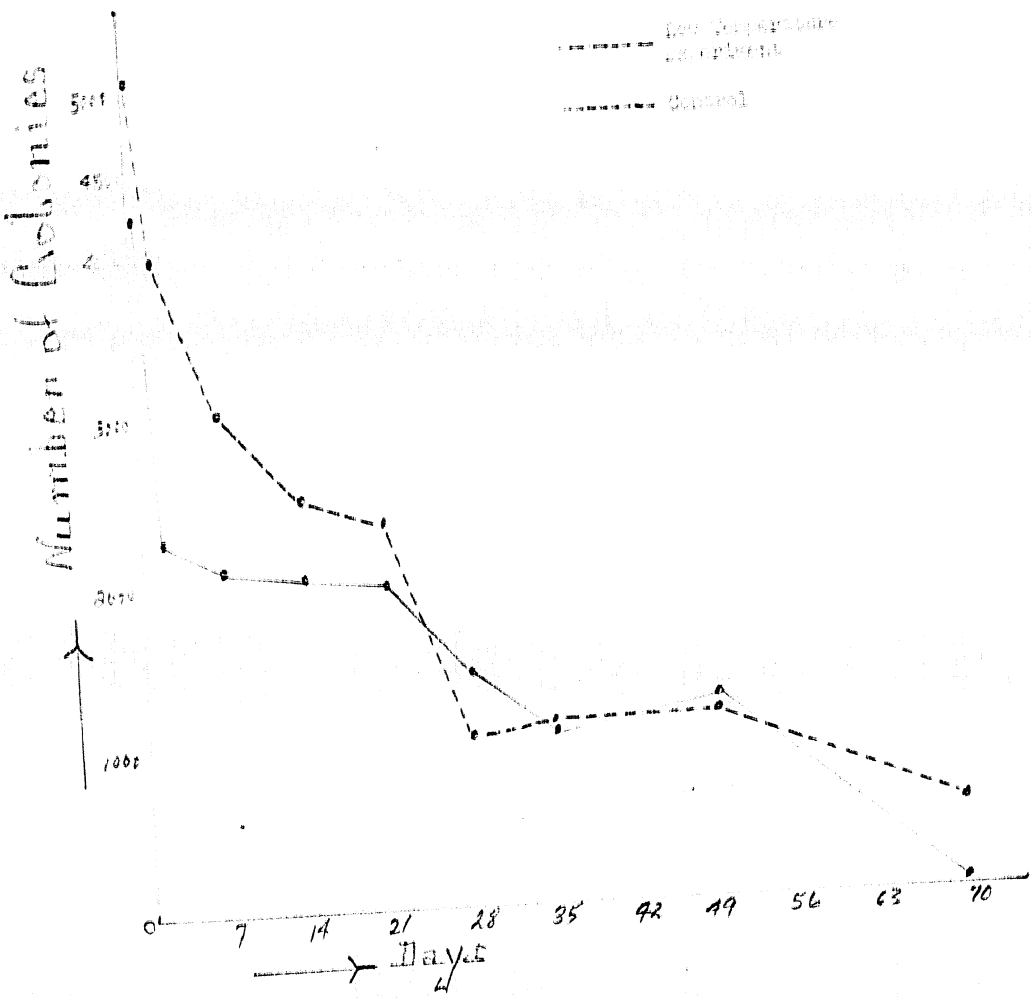
PAGE NO. 16

EXPERIMENTAL CURVE FOR BACILLUS ANTHRACIS SPORULATION

Experiment in the Low Temperature Laboratory in which Bacteria were Placed Without Delay Removed From the Culture Media.



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Experiment No 2

The results of the effect produced when sterile saline suspensions are subject to low temperature are indicated in Table 6.

Bacillus dysenteriae Shiga type:

This produced many colonies which were sufficiently identified and counted on the 21st day. After 28 days, the colony number decreased drastically until the 84th day. All died by the 105th day. In the case of the control cultures many colonies were produced which could not be counted until the 28th day, but growth began to suddenly cease after 49 days. This means that those exposed to room temperature did not survive long as those subjected to low temperature.

Bacillus dysenteriae Kowajone A

The resistance of this bacteria were roughly similar to Bacillus dysenteriae Shiga type. In the control, it produced just enough colonies to be counted. After that the number suddenly decreased in arithmetic progression each day. There were still some produced after 63 days, but all died by 105 days. On the contrary, those subjected to ordinary room temperature produced many colonies during the last period, but suddenly lost their viability and died in 35 days.

Bacillus dysenteriae Kowajone D

The resistance displayed by this organism to low temperature was stronger than that indicated by Bacillus dysenteriae Shiga type and Kowajone A. The colony number decreased suddenly after 21 days, to less than 4 percent of the original number. Many colonies still existed on the 84th day, and some even lived for 105 days.

On the other hand, the control at room temperature produced so many colonies they could not be counted. Although the number decreased to some extent after 70 days, there were still many colonies after 105 days.

Bacillus megaterium infectum.

This bacteria showed colony production actually after 14 days, but the decrease was less after that. On the 22nd day, the number equalled about one-fourth of the number present on the 7th day. They took their virtually completely in 105 days. On the contrary, there in the control produced many colonies without any significant change even after 105 days.

TABLE NO 6

EXPERIMENT NO 2

Low Temperature Experiment with Sterile Saline Emulsion.

	Bacillus dysentiae Shiga type		Komagome A		Komagome B		Bacillus dysentiae infantum (Ohara-Minota Bacillus)	
	Low Temperature	Control	Low Temperature	Control	Low Temperature	Control	Low Temperature	Control
Immediately Before the Experiment	Many	Many	Many	Many	Many	Many	Many	Many
12 Hours	More Than 100,000	Many	Many	Many	Many	Many	Many	Many
24 Hours	More Than 100,000	Many	Many	Many	Many	Many	Many	Many
2 Days	More Than 100,000	Many	Many	Many	Many	Many	16962	Many
4 Days	More Than 100,000	Many	More Than 100,000	Many	Many	Many	16245	Many
7 Days	More Than 100,000	Many	More Than 100,000	Many	More Than 100,000	Many	14746	Many
14 Days	More Than 100,000	Many	7629	Many	More Than 100,000	Many	5085	Many
21 Days	About 100,000	Many	1017	1695	4520	Many	5085	Many
28 Days	3559	Many	828	4	3729	Many	3955	Many
35 Days	3360	Many	791	0	3650	Many	2825	Many
49 Days	3153	0	481	0	2934	Many	2685	Many
70 Days	3059	0	248	0	2542	Many	2542	Many
84 Days	2812	0	200	0	2425	Many	2373	Many
105 Days	0	0	0	0	640	Many	0	10170

TABLE NO 6

EXPERIMENT NO 2

Low Temperature Experiment with Sterile Saline Emulsion.

	Bacillus dysentiae Shiga type		Komagome A		Komagome B		Bacillus dysentiae infantum (Ohara-Minota Bacillus)	
	Low Temperature	Control	Low Temperature	Control	Low Temperature	Control	Low Temperature	Control
Immediately Before the Experiment	Many	Many	Many	Many	Many	Many	Many	Many
12 Hours	More Than 100,000	Many	Many	Many	Many	Many	Many	Many
24 Hours	More Than 100,000	Many	Many	Many	Many	Many	Many	Many
2 Days	More Than 100,000	Many	Many	Many	Many	Many	16962	Many
4 Days	More Than 100,000	Many	More Than 100,000	Many	Many	Many	16245	Many
7 Days	More Than 100,000	Many	More Than 100,000	Many	More Than 100,000	Many	14746	Many
14 Days	More Than 100,000	Many	7629	Many	More Than 100,000	Many	5085	Many
21 Days	About 100,000	Many	1017	1695	4520	Many	5085	Many
28 Days	3559	Many	828	4	3729	Many	3955	Many
35 Days	3360	Many	791	0	3650	Many	2825	Many
49 Days	3153	0	481	0	2934	Many	2685	Many
70 Days	3059	0	248	0	2542	Many	2542	Many
84 Days	2812	0	200	0	2425	Many	2373	Many
105 Days	0	0	0	0	640	Many	0	10170

GRAPH NO 17

LYMPHOCYTICAL CELLS IN BACILLUS DYSENTERIAE CARRIER

Low Temperature Incubation with Sterile Saline Isolation.

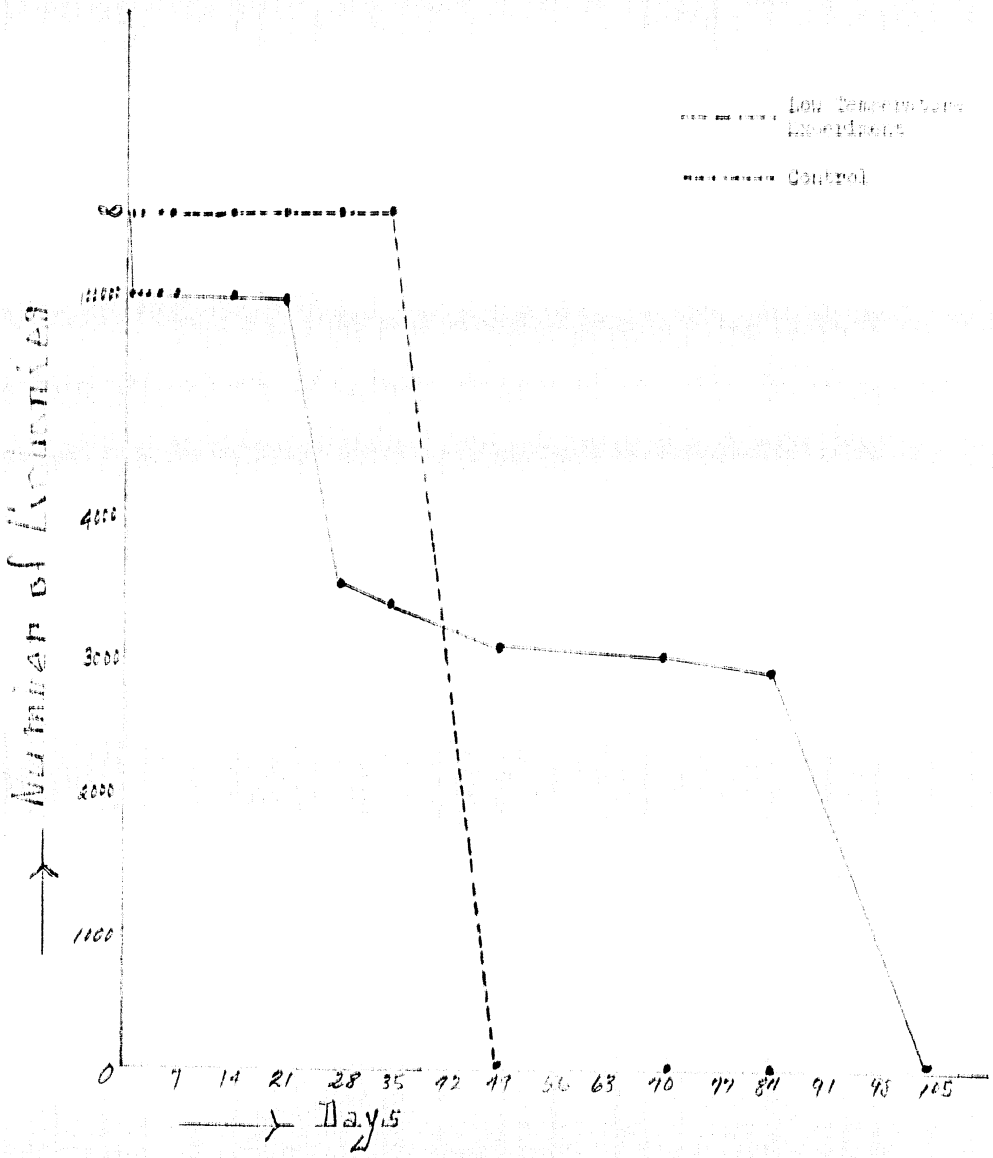


CHART NO 18

EXPERIMENTAL GERMS FOR INSAPOR A

Low Temperature Experiment with Dracile Saline Emulsion.

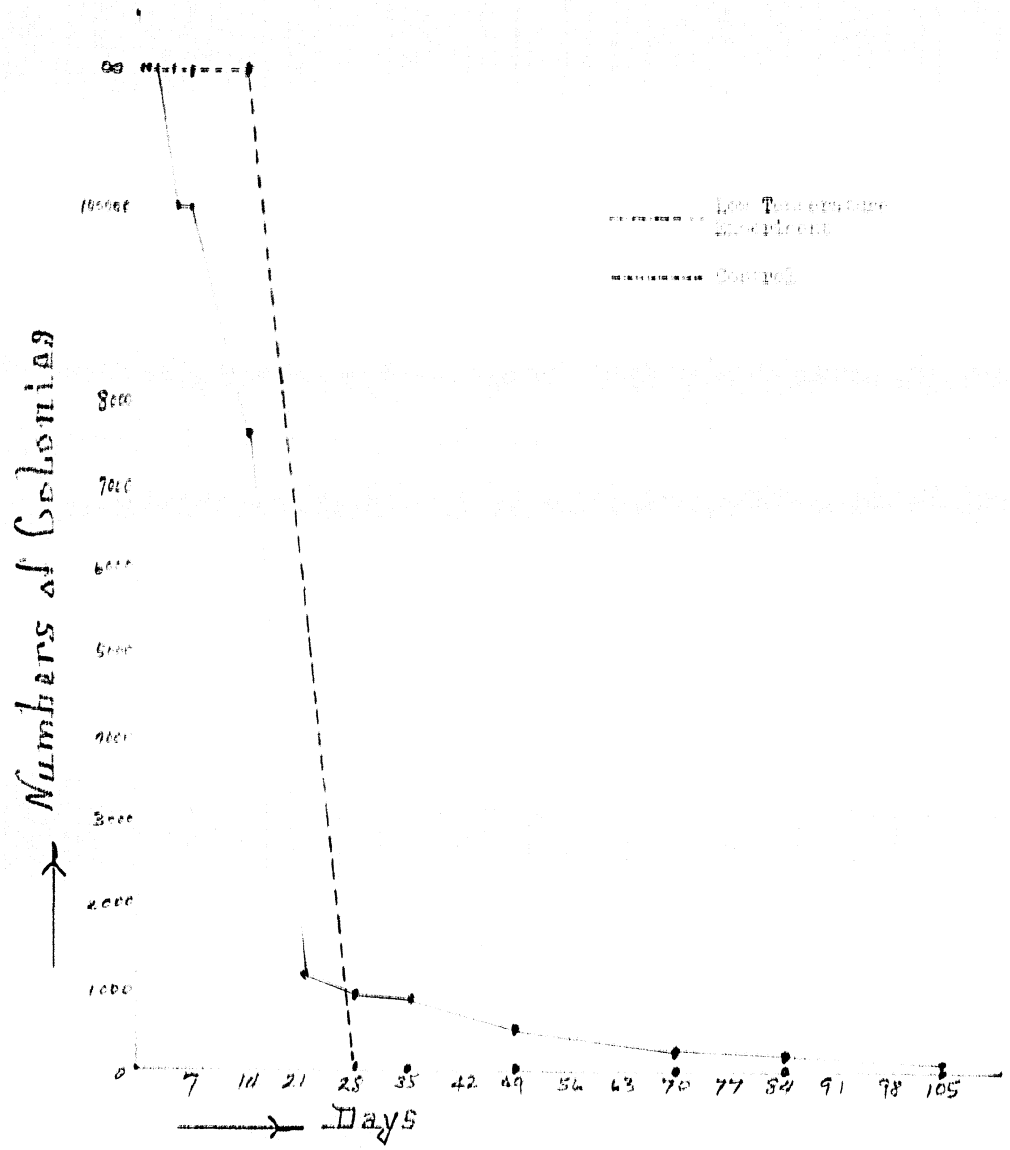


PLATE NO 19

EXPERIMENTAL CHART FOR MONTHLY B

Low Temperature Experiment with Sterile Saline Emulsion.

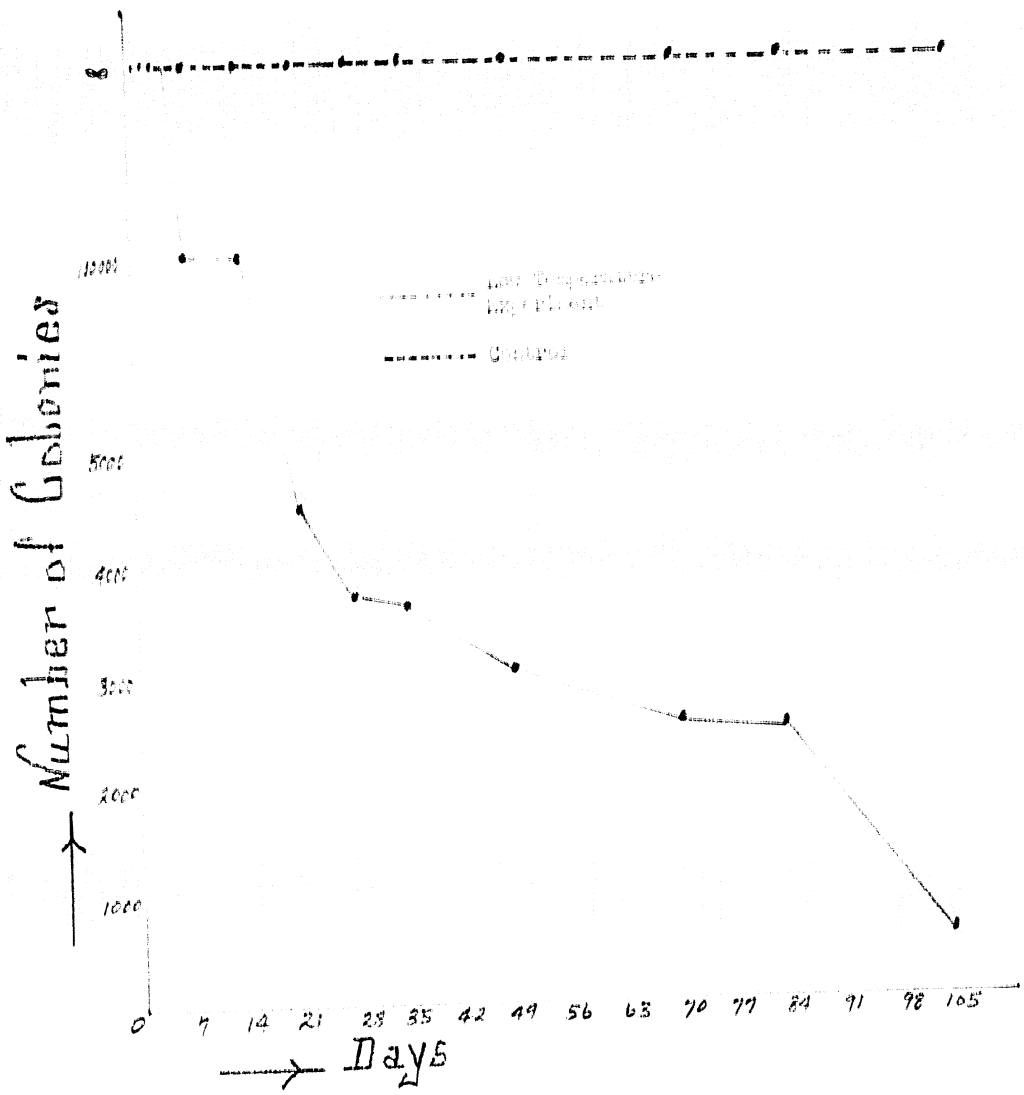
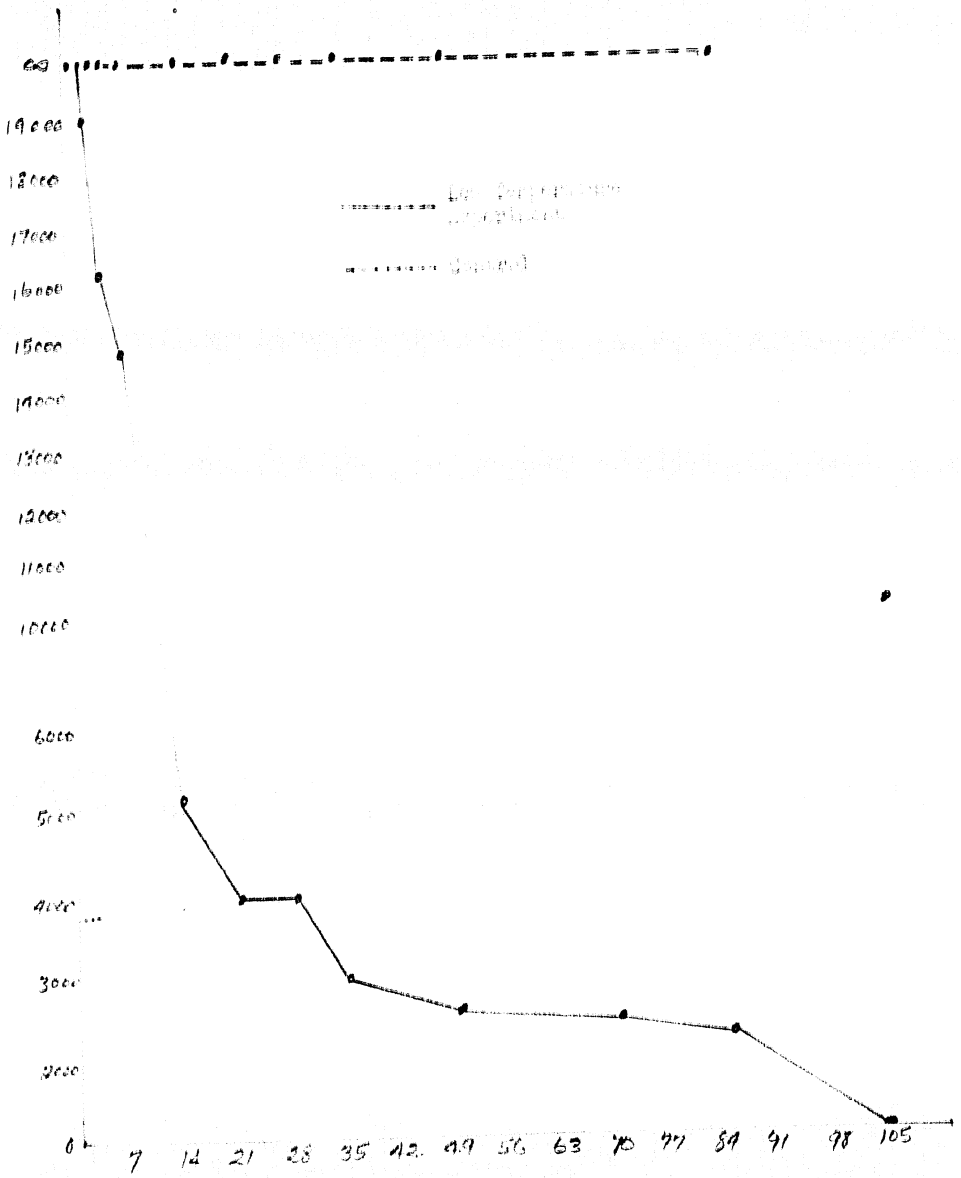


FIGURE NO. 29

LOGARITHMIC GROWTH OF BACILLUS MELITENSIS ER

[illegible]



Summary of Item 2

In summary, the resistance of these 4 bacteria was proven to be rather strong. As illustrated from the experiments in the low temperature laboratory where bacteria in their culture media were placed, Bacillus dysenteriae Shiga type had resistance on the strongest. The colony number was almost the same as the original number even after 70 days. In the case of Komagome A, the colony number decreased gradually during the first 7 days, but it showed little change after that. In the case of Komagome B, the resistance existed, although the colony number was less than one thousand. In the case of Bacillus dysenteriae infantum, the colony decreased gradually. Although [illegible] were still some colonies of bacteria, even after 70 days, in the larger 1 bacteria. In the [illegible] bacteria as from temperature which each [illegible] in the [illegible] of the experiment. At the same time, the colony number had not been reduced, further than that of the low temperature.

Moreover, from the above statement, that the specific saline solution does not harm the experiment. These 4 bacteria under deep temperature exposure produced similar amounts of colonies for a relatively long period. Bacillus dysenteriae infantum increased in colony production suddenly after 105 days and Komagome B after 70 days. Bacillus dysenteriae Shiga type died out suddenly after 49 days and Komagome A after 35 days. These bacteria which were exposed to low temperature were affected to a similar degree and colony production decreased gradually. Among these 4, the resistance of Komagome B, which survived in small numbers even after 105 days, was the strongest. The resistance of Bacillus dysenteriae, Komagome A, and Bacillus dysenteriae infantum was almost similar, while all 4 types died in toto by 105 days.

From the above observation, it was noticed that the resistance of these 4 bacteria to low temperature such as minus 30 degrees Centigrade was very strong though there were varying differences. They could survive for 70 to 80 days. When exposed to low temperature while still in the culture media, it is known that the effect of surface dryness is more serious.

In these experiments, Bacillus aquacolonicus chlorogenus [illegible], [illegible] sterile saline solution condition, and Bacillus aquacolonicus [illegible] at low temperature. [illegible] similar results. It is noted that Bacillus aquacolonicus in a sterile-water condition survived for 30 to 40 days at low temperature but lived only 7 days at room temperature. Nevertheless, it could not exceed 14. As mentioned, it is a fact that Bacillus aquacolonicus [illegible], [illegible] 4 and 5, and Bacillus aquacolonicus infantum possess a rather strong resistance especially to low temperature.

Item 2. The Effect of Low Temperature on Staphylococcus

Staphylococcus is the strongest pleurococcus among those not forming spores. Its vitality lasts for a long period under dry conditions. Staphylococcus applied to papers lived for 110 days at room temperature and for 70 days at irradiation temperature (Sechongchang). It is stated that Staphylococcus applied to silk thread or linen does not die for 3 and ½ months to 5 or 6 months or even more (Hirstein). Accordingly, its resistance to low temperature is also strong. According to Ha. Weisser, it was impossible to kill Staphylococcus even after scores of repeated alternations of freezing and melting under minus 23 degrees Centigrade.

Prudden (1887) made an emulsion with Staphylococcus citreus and sterile water, and froze it by exposing it to outdoor temperatures rang-

ing from minus 1 to minus 11 degrees Centigrade. He found there were incalculable numbers of bacteria for 66 days even after the freezing procedure while the same bacteria on the surface of the old and dried meat died 7 days after freezing.

[illegible] (1900) used various methods of exposing to liquid air for 15 minutes, the species of the staphylococcus citreus and bacillus anthracis, but he could not change their properties.

Park (1902) stated that staphylococcus citreus survived even after being exposed to liquid air for 120 minutes. Rudolph (1904) stated that he put staphylococcus citreus in liquid air for 6 months but he could find no change in its vital functions, although its ability to form pigment and produce toxin, its substances had been destroyed.

Ward and Krall (1907) applied staphylococcus thinly to the surface of a shell and placed it at room temperature, then refrigerated it at the low temperature of minus 190 degrees Centigrade. On the 1st day most of the bacteria died. After that a small number of colonies were produced without any new variation. When the low temperature of minus 183 degrees Centigrade was used some survived even after 100 days. At room temperature, all died after 5 days. When refrigerated, most died after 100 days.

In Japan, Arai (1931) declared that Staphylococcus endured after 60 days when refrigerated between minus 6 and minus 11 degrees Centigrade, and that the degree of development dropped to 50 percent of the original on the 69th day and to 30 percent after 78 days, but did not die out completely. Kasuga and Matsutani stated that Staphylococcus still possessed vitality even after being exposed to the temperature of minus 30 degrees Centigrade for 30 days.

[redacted]

My experiment with the low temperature laboratory at about minus 30 degrees Centigrade indicates various effects on Staphylococcus as follows.

Experiment No 1

Staphylococcus citreus, Staphylococcus albus and Staphylococcus aureus were cultured in ordinary agar slants, exposed to the low temperature of about minus 30 degrees Centigrade and examined to see whether they were still living or dead.

Staphylococcus citreus and Staphylococcus albus

Both of these bacteria, at the low temperatures indicated in Table 7, show that the number of colonies drastically decreased, after 3 weeks, to five-eighths of the number present the first day of the experiment. Thereafter, the number remained, generally, and after 35 days only two-fifths or one-half of the original colonies remained. They never died out completely and many colonies were produced from these remnants. In the case of the bacteria which were exposed to room temperature, the bacterial mass on the culture medium's surface lost their moistness and stuck to the surface as it became drier each successive day. Thus, the process of obtaining a loopful became difficult. The colony number had decreased drastically by the 7th day. In the case of Staphylococcus citreus, the number decreased to one-fifth of the original; and in the case of Staphylococcus albus, to one-seventh. Then, the rate of death increased tremendously and the colony number decreased from one-fortieth to one-sixtieth of those which underwent low temperature exposure.

Staphylococcus aureus

Here the results were almost similar to those involving the above 2 bacteria. The colony number decreased gradually at the low tem-

perature, but even after 70 days one-fourth of the colony number existing the seventh day was still present. On the other hand, those subjected to room temperature died almost entirely by the fifth day and decreased to one-eighteenth of the number existing the seventh day. After 70 days, only a portion possessed their vitality.

These results corresponded to those obtained by predecessors, who already knew that the capacity to resist low temperature is stronger, but the capacity to resist unsuitable conditions is far weaker than that [illegible] [illegible].

TABLE NO 7

EXPERIMENT NO 1

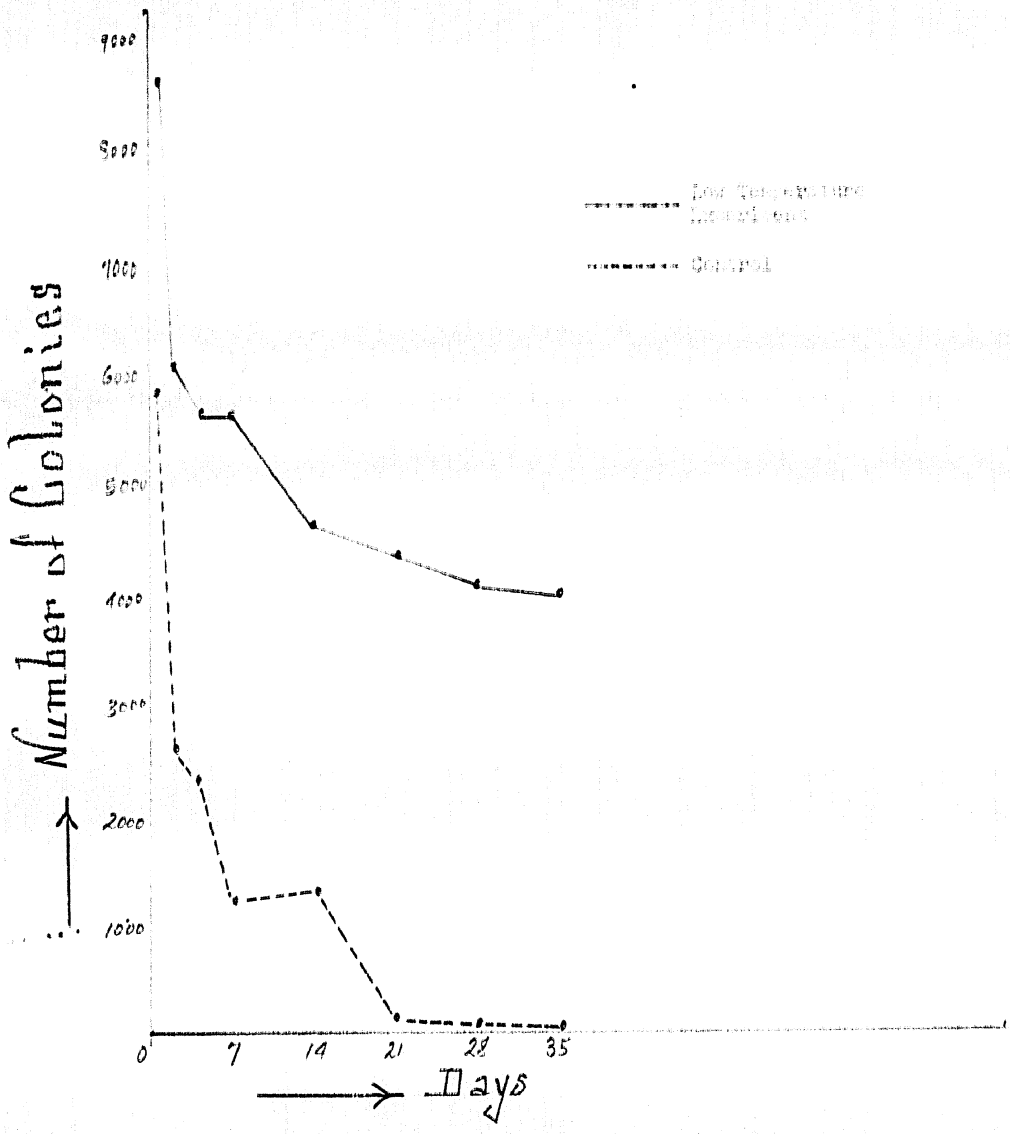
Experiment in the Low Temperature Laboratory (around minus 30 degrees Centigrade) in which Bacteria Were Placed Without Being Removed From the Culture Media.

	Staphylococcus Citreus		Staphylococcus Albus		Staphylococcus Aureus	
	Low Temperature	Control	Low Temperature	Control	Low Temperature	Control
1 Day	[illegible]	[illegible]	2359	[illegible]		
3 Days	6058	[illegible]	[illegible]	7130		
4 Days	6550	2316	2853	1674		
7 Days	6534	1343	3407	508	10272	17272
14 Days	4637	1356	3356	305	10272	6367
21 Days	4390	194	2056	284	15343	1113
28 Days	4151	174	1695	174	12350	208
35 Days	4068	158	1921	31	12221	290
45 Days					11187	156
70 Days					4955	35

GRAPH NO 21

EXPERIMENTAL CURVE FOR STAPHYLOCOCCUS CITREUS

Experiment in the Low Temperature Laboratory in Which Bacteria Were Placed Without Being Removed From the Culture Media.



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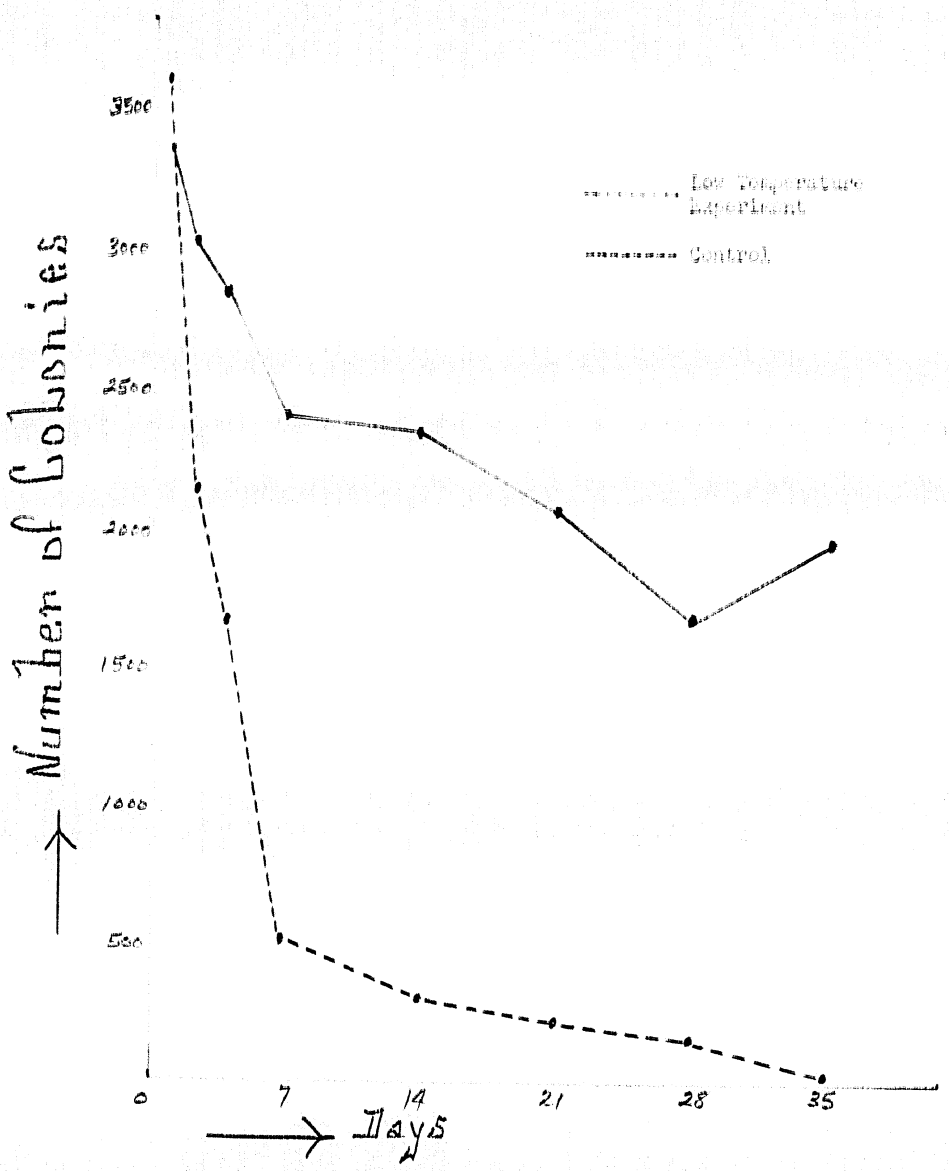
[redacted]

EXPERIMENTAL CURVE FOR STAPHYLOCOCCUS ALBUS

Encountered in the Low Temperature Laboratory in which Bacteria Were Placed Without Being Removed from the Culture Media.



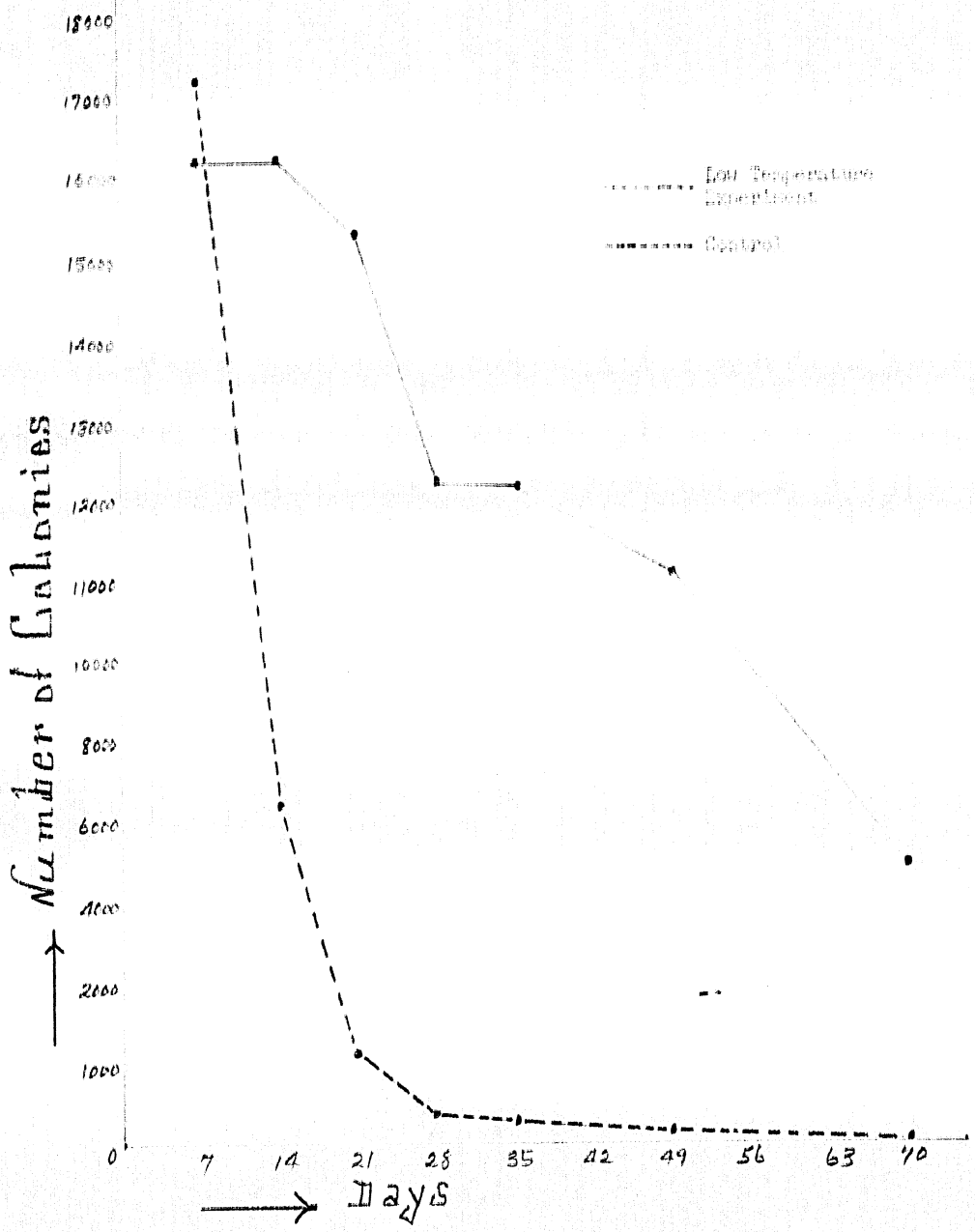
- RESTRICTED

RESTRICTED

GRAPH NO 23

EXPERIMENTAL CURVE FOR STAPHYLOCOCCUS AUREUS

Experiment in the Low Temperature Laboratory in which Bacteria were placed without being Removed from the Culture Media.



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Enclosure No. 1

When the saline suspension of staphylococcus aureus underwent low temperature treatment, the effect was found to be negligible and the colony number obtained on the 70th day was almost the same as the original number. Although the number decreased suddenly after that, some colonies were still produced after 105 days. On the contrary, those substituted at room temperature produced almost the same number as the original up to the 21st day, but then suddenly decreased in production and died out on the 70th day. (See Table 2).

In summary, the resistance of staphylococcus aureus was relatively good in the saline solution, but its very strong resistance for temperature when frozen in the form of suspension of the cells survived for 105 days.

TABLE NO 8

EXPERIMENT NO 2

Low Temperature Experiment with Sterile Saline Emulsion.

Staphylococcus Aureus

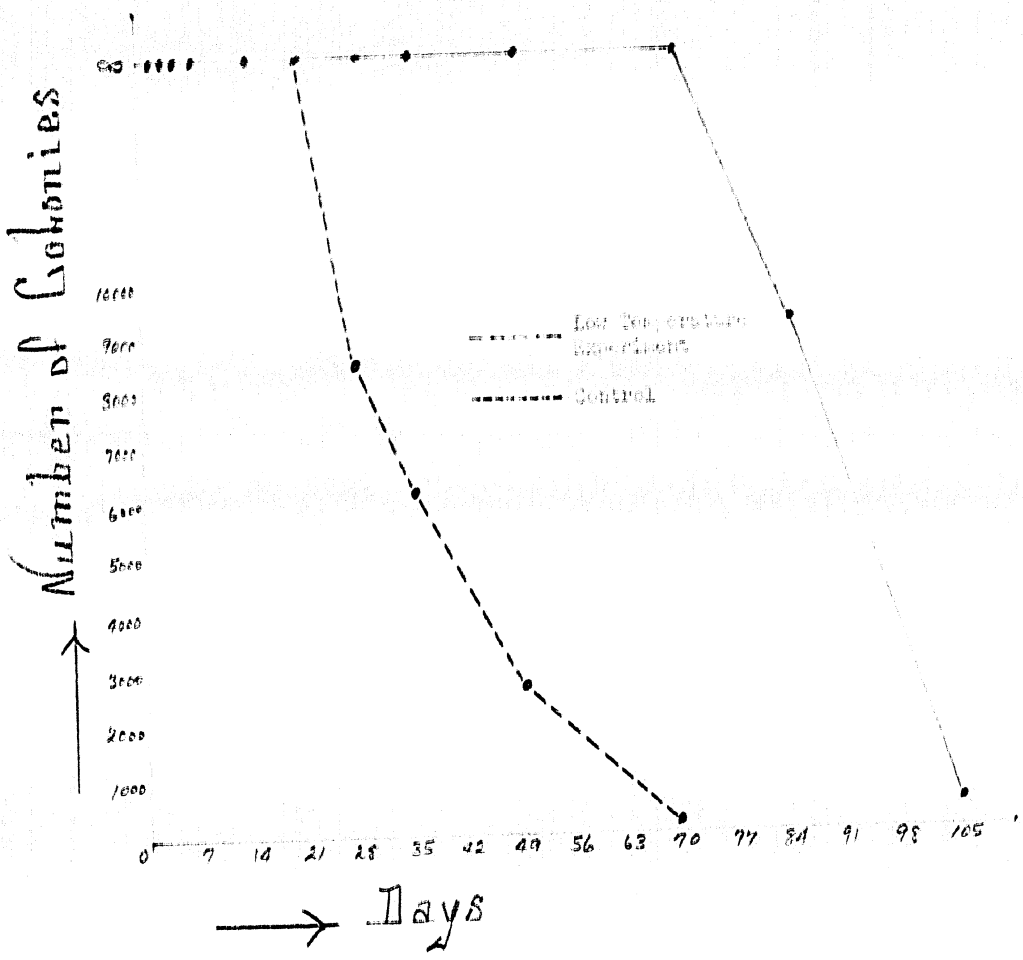
Immediately After	Low Temperature	Control
1 Day	Lang	Lang
3 Days	Lang	Lang
4 Days	Lang	Lang
7 Days	Lang	Lang
14 Days	Lang	Lang
21 Days	Lang	Lang
28 Days	Lang	7550
35 Days	Lang	6110
49 Days	Lang	9542
70 Days	Lang	0
84 Days	9053	0
105 Days	453	0

RESTRICTED

GRAPH NO 24

EXPERIMENTAL CURVE FOR STAPHYLOCOCCUS AUREUS

Low Temperature Experiment with Sterile Saline Emulsion.



RESTRICTED

Summary of Item 4

The resistance of Staphylococcus to the low temperature of minus 30 degrees Centigrade is very strong. When exposed to the low temperature in a culture media, most of the colonies were found survived even after 35 to 70 days, although the colony number decreased to some extent. The colony number of Staphylococcus aureus in the saline solution suspended [illegible] after being exposed to low temperature (ice) for 70 days. [illegible] that the viability decreased considerably. The colonies did not [illegible] the [illegible] 14 days, but only a portion of them survived.

On the contrary, [illegible] low temperature led to a reduced production of [illegible] days. Although the bacteria survived during the 35 to 70 day period, they produced less colonies. This is the first resistance to dryness in inverse relationship to the resistance offered to low temperature. Moreover, it was found that the resistance of the Diplococcus pneumoniae in saline suspension at room temperature became relatively weaker as the bacteria dies out in the 70 day period.

Item 5 The Effect of Low Temperature on Diplococcus Pneumoniae

The resistance of Diplococcus pneumoniae to dryness is extremely weak when the bacteria is on the surface of the culture media or when it is applied to a thin blood smear (Kruse, Panzini). The resistance to both dryness and light is considerable when it is contained in a protein albuminous substance such as blood or sputum. According to Guarnieria, Diplococcus pneumoniae survived for at least 1 month when the blood containing this bacteria was attached to a feather and was dried in a drier.

According to Forbodie and Lifreduzzi, this bacteria survived in dried sputum for 14 to 55 days. According to Serano, this bacteria contained in the spleen and tissue of pneumonia patients in sterile dust survived for 120 days to 180 days. Potter proved that this bacteria existed in the weighing dusts of hospital air for 21 days. Grundmann stated that Diplococcus pneumoniae attached to a principle live from 70 to 90 days, but he also stated that Diplococcus pneumoniae, mixed in blood or sediment matter, survived for the longer period of about a year when exposed to a cold climate.

According to [illegible], Serano, [illegible] of Diplococcus pneumoniae [illegible] culture [illegible] Diplococcus pneumoniae [illegible] by the [illegible] of the [illegible] according to the bacterial [illegible]. [illegible] survived usually about 6 to 12 days [illegible] but [illegible] for 4 to 60 days.

[illegible] pneumoniae [illegible] virulence for a longer period if [illegible] preserved in the [illegible] (Serano, Pacella). Under the circumstances, the [illegible] to be [illegible]. According to Serano and Lubatoni, this bacteria [illegible] [redacted] in 19 days under a temperature of [illegible] 25 degrees Centigrade.

The culture medium, into which the Diplococcus was transplanted, was placed in the low temperature laboratory at minus 20 degrees Centigrade. The results of the experiments on mutations are as follows:

Three experiments were made with Diplococcus pneumoniae Type I. Those exposed to low temperature maintained their vitality longer than those exposed to room temperature. During the last experiment, the colony number produced after low temperature exposure for 15 days was one-third the original; while the colony number at room temperature decreased to

about one one-hundredth in 1 week, to one two-hundredth in 2 weeks, and to one three-hundredth in 3 weeks. On the 30th day, all were dead and not a single colony was produced. During the third experiment, colonies were actually produced in the low temperature laboratory. After 70 days, ½ of the original colony number was produced. Those exposed to room temperature were dead in a week.

The culture of M. tuberculosis Strain H37Rv was [illegible]. In the 1st experiment, the resistance to low temperature was shown to be very great. About ½ the original colony number was produced in 3 weeks and about one-hundredth as many in 4 weeks. Those exposed to room temperature died in 7 days only. During the 2nd experiment, the results were the same. One-half of the original colony number was produced in 2 weeks at low temperature, and one-tenth was produced in 4 weeks. On the other hand, those that had been exposed to laboratory temperature as a control survived for only 7 days [illegible] the 1st experiment.

Summary of Items

It is clear that both M. tuberculosis Strain I and H37Rv exposed to room temperature or incubation died out faster than those exposed to a low temperature of minus 30 degrees Centigrade.

Colonies of M. tuberculosis pneumoniae on the blood agar slants were transparent and small at first, resembling dew-drops. Then they became flat and the blood pigment in the culture media changed to a dark green color. Consequently, the underlying layers of the colonies looked dark green when observed through the culture media. Each successive day the culture media became paler, and the colonies gradually lost their dew-drop shape and their luster. After 7 to 10 days, the colony existence became obscure, and only the blood pigment which had become dark green survived forming a sharp point. Consequently, it became extremely dif-

fault to rich for colonies after 7 days. On the contrary, thin ice was formed on the plastic surface of culture media exposed to low temperature. When this was transferred from the low temperature laboratory to room temperature, the surface of ice began to melt within 4 or 5 minutes. Thus there was no difficulty in picking out colonies.

Of course, darkness was the most important factor causing the relatively short life of bacteria at room temperature, but White and Penny attributed the result to the acid formed by chlorine compounds.

Wendt and Porter recommended preserving [illegible] in the refrigerator. In this experiment, the bacteria exposed to the low temperature laboratory at about 20 degrees Centigrade lived for 70 days. The results prove that the bacteria is extremely resistant to low temperature.

TABLE NO 9

Experiment in the Low Temperature Laboratory (around minus 30 degrees Centigrade) in which Bacteria Were Placed Without Being Removed From the Culture Media.

	Diplococcus pneumoniae Typus I						Diplococcus pneumoniae Typus II			
	First Experiment (5/III 9/IV)		Second Experiment (14/III 9/V)		Third Experiment (1/IV 10/VI)		First Experiment (2/III 26/III)		Second Experiment (4/IV 2/V)	
	Low Temperature	Room Temperature	Low Temperature	Room Temperature	Low Temperature	Room Temperature	Low Temperature	Room Temperature	Low Temperature	Room Temperature
1 Day	3559	3542	4230	4400	4237	5819	4520	1525	5678	4565
2 Days			4186	4210	4051	3277	3559	791	4532	3456
3 Days	3390	850					3051	567		
4 Days			4621	60	4110	450	2825	100	3675	2356
5 Days	3390	68					2325	50		
7 Days	3085	27	3390	20	4019	320	2245	4	3051	20
14 Days	3051	17	1621	0	2542	0	2125	0	2565	0
21 Days	2308	10	1525	0	2320	0	2050	0	2250	0
28 Days	1525	5	1050	0	2235	0	1200	0	4800	0
35 Days	1525	0	0	0	2242	0			0	0
42 Days			0	0	2238	0			0	0
49 Days					1500	0				
56 Days			0	0	1424	0				
70 Days					2034	0				

IV GENERAL SUMMARY AND CONSIDERATIONS

Low temperature experiments were conducted on various bacteria as previously enumerated. The conclusion reached in these experiments was that the resistance capacity of various bacteria is quite high to low temperature and it was impossible to kill them in a short period. When they were frozen at low temperature without being removed from the culture media or at low temperature in saline solutions, no complete bacterial destruction could be found before 70 days. When using saline solutions, in the experiment, Bacillus proteus vulgaris, and Bacillus typhosus displayed the weakest resistance to low temperature of all bacteria used in the experiments. They died out after 24 days. The resistance of Bacillus pyocyaneus origin type and Bacillus pyocyaneus infantum was somewhat stronger. They were able to survive in their entirety for about 105 days. Bacillus coli, Bacillus paratyphosus A and B, Bacillus dysenteriae Shiga A and B, and Staphylococcus aureus were the strongest and lived for more than 105 days.

It has been proven by many scholars in the past that the resistance capacity of various bacteria to low temperature is exceedingly high, but since most of them employed outdoor exposures in their experiments, discrepancies in the temperature at different times were present. Consequently it was impossible to operate below a certain degree in low temperature for a number of days. There were experiments in which the bacteria was exposed to the extremely low temperature of minus 190 degrees Centigrade, such as produced by liquid air, but these were not practical.

However, the low temperature equipment in the low temperature laboratory was maintained at a constant degree, similar in all respects to the natural environment, by using compressed ammonia gas. And, an advan-

once it gradually operating at a constant temperature.

From the results of experiments on various bacteria, it was generally observed that most of the colonies died in a short period and the most gradually [illegible] after that short period. The exception to this was that [illegible] the small number of colonies [illegible], but lived for long periods. [illegible], Hohlen and [illegible], [illegible], and [illegible], etc. made detailed investigations and reports on this fact.

[illegible] (1904) [illegible] [illegible] percent of [illegible] [illegible] [illegible] (1903) [illegible] that [illegible], [illegible] the bacillus [illegible] after being frozen, and [illegible] percent of the bacillus [illegible]. They [illegible]. In [illegible], that [illegible] 50 percent of the bacillus [illegible]. In [illegible], [illegible] percent of [illegible] bacteria [illegible] lived for a long [illegible] in the ice in [illegible] water. [illegible] bacillus [illegible] maintained its vitality for 2 months; and bacillus [illegible] for [illegible] months, were 2 other [illegible] they observed.

[illegible] and [illegible] (1907) [illegible] staphylococcus to the surface of a shell, dried it, and exposed it to room temperature, and then refrigerated it at the low temperature of minus 183 degrees Centigrade. They found that most of the colonies died on the 1st day, then a small number of the colonies were regularly produced without too much change. In the case where this organism was exposed to a low temperature of minus 183 degrees Centigrade, the colonies survived even after 100 days; when ex-

[illegible] to room temperature, all colonies were dead in 5 days; and when refrigerated [illegible] all were dead in 150 days.

There were [illegible] differences in these results according to the bacterial species, the type of medium, and the bacterial number, but they were generally [illegible] [illegible] their survival periods [illegible].

[redacted]

[illegible] Consequently, as indicated in cases of Bacillus [illegible], which [illegible] to their original numbers [illegible] [illegible].

Therefore, it is in accord with [illegible]'s theory that bacteria in a nourishing medium such as a culture medium do not easily perish from cold exposure since they are able to obtain some nourishment although the activity of the organism is slowed down resulting in reduced absorption of nutrients. Contrary to this, bacteria in media such as water or saline solution die out in a short time since their biological functions are retarded and the nutritive media become scarce. Thus only those bacteria actively functioning can survive and live for long periods.

When the control was placed in the low temperature laboratory without being removed from the culture media, that is to say which had been exposed to room temperature, the harvests on the culture media's surface became dry, despite the fact that they were especially cotton-stoppered and paraffined. This is the reason why colony numbers were usually less than those obtained at low temperature. It has been proven especially that staphylococcus died out after 7 days. It is clear from this that the resistance capacity exhibited by bacteria exposed to dryness is relatively weak, while the resistance capacity at low temperature is quite strong.

Usually the concepts were [illegible] that [illegible] in the [illegible] drop in temperature. When we tested the cells drawn at low temperature, the [illegible] colony [illegible] 32 to 34 [illegible].

Nevertheless, experiments were conducted without testing those which had been frozen at low temperature. They were [illegible] room temperature (18 to 20 degrees Centigrade). Consequently, no [illegible] [illegible] period of [illegible] within [illegible].

V Conclusions

(1) Experiments were conducted on the effect of low temperature of about minus 30 degrees Centigrade on the development of Bacillus coli, Bacillus pyocyaneus, Bacillus proteus vulgaris, Bacillus typhosus, Bacillus paratyphosus A and B, Bacillus dysenteriae shiga type, Bacillus dysenteriae Sonnei A and B, Bacillus dysenteriae Inaburu, Streptococcus, and Diplococcus pneumoniae in the Northeast low temperature laboratory attached to this University.

(2) The methods used to produce cold endurance on the above stated various bacteria were:

(a) The bacteria, cultured for 24 hours at 37 degrees Centigrade on ordinary agar plates (in the case of Diplococcus pneumoniae, blood agar plates were used) were placed in the low temperature laboratory without removing them from the culture media. They were discovered from the low temperature laboratory after 1, 2, 4, 7, 14, 21, 28, 35, 49, and 70 days and the number of colonies developed were counted;

(b) Bacteria, cultured for 24 hours at 37 degrees Centigrade on ordinary agar plates were diluted with saline solution to form bacterial suspensions. They were then put into the low temperature laboratory after being poured into several solid test tubes. They were then removed from the low temperature laboratory after 12 hours and 1, 2, 4, 7, 14, 21, 28, 35, 49, 70, 84, and 105 days and the number of colonies counted. The above stated 2 methods were used. In each case, the control was placed at room temperature and the culture media was carefully paraffined.

(3) The resistance of various bacteria to low temperature is very strong. In the case of those bacteria transplanted into the culture media without being removed from the media, some colonies were produced,

though in small numbers, even after 70 days. In the case of those made into a suspension with saline solution and exposed to low temperature, as for example those which were exposed to low temperature without being taken out of the culture media, none of the bacterial culture died out within 70 days. Bacillus pyocyaneus, Bacillus proteus vulgaris, and Bacillus typhosus did not die out until the 105th day. There were still some colonies produced after 105 days in the case of Bacillus coli, Bacillus paratyphosus A and B, Koragome A and B, and Staphylococcus aureus.

(4) With regard to both Diplococcus pneumoniae Type I and II cultured on blood agar media, those that were placed at room temperature did not produce any colony on the 14th day, while those exposed to low temperature exhibited a strong resistance. Type I survived for from 56 to 70 days or more, and Type II survived for 70 days and produced colonies.

(5) When Bacillus coli, Bacillus pyocyaneus, and Bacillus proteus vulgaris, were subjected to low temperature or were without being taken from the culture media, the number of the produced colonies drastically decreased within 7 days. Although some produced only a small amount of colonies to maintain the overall number, most of them began decreasing in the number of colonies produced day after day. Paradoxically, although the culture media were paraffined, due to their gradual drying those control bacteria exposed to room temperature without being removed from the culture media produced fewer colonies from day to day. Also most of them showed a sudden decrease in a short period compared to those which had been exposed to low temperature.

(a) Bacillus coli, Bacillus typhosus, Bacillus paratyphosus B, Bacillus dysenteriae Shiga type, Koragome B, and Staphylococcus at room

temperature produced less colonies by a suddenly decreasing process, while they also underwent a gradually decreasing process with a relatively smaller decrease in colony production when they were exposed to low temperature. The conclusion is that those exposed to low temperature always produced more colonies than those exposed to room temperature.

(b) Bacillus megaterium, Bacillus proteus vulgaris, and Bacillus A, at first suddenly decreased in production of colonies when they were exposed to low temperature and then maintained a level of production, but small in number of colonies. On the other hand, those exposed to room temperature suddenly decreased in colony production, but after 30 to 40 days they produced colonies thereafter and became even better in number than those produced by those exposed to low temperature. This fact can be explained by the nature of the culture media when placed at room temperature.

(c) The decrease in the number of produced colonies of Bacillus [illegible] subtilis after low temperature exposure was the same as after room temperature exposure, and they decreased in arithmetical progression.

(d) Bacillus bipolosus A at room temperature always produced more colonies than when placed at low temperature. The rate of production was always similar and the number of colonies decreased in a like manner.

In summary, the resistance capacity of the above stated bacteria was very high even after being exposed to low temperature of about minus 30 degrees Centigrade for a long period and they were able to produce colonies even after many days. On the contrary, those cultured at room

[redacted]

temperature reduced colony production after several days as the surface of the culture media became dry. From the above facts, we can deduce that the dryness of the culture media had a far worse effect on the vital capacity of bacteria than any extremely low temperature.

( ) Some bacteria showed less susceptibility with saline and exposed to low temperature, decisively reduced their production of colonies while a great many, although there were differences. After that, not such marked decrease was exhibited in the number of colonies and a considerable number of colonies of bacteria survived for a long period of time.

(a) Bacillus tuberculosis and Bacillus coli showed marked effects from the effect of low temperature exposure and were more than 90 percent dead in 7 days.

(b) Bacillus coli, Bacillus typhosus, Bacillus paratyphosus A and B, decreased in colony production to half the original number after 1 or 2 days. After 14 to 21 days, the number of colonies decreased to less than 10 percent of the original.

(c) Bacillus pseudomonas aeruginosa, Komagome A and B, and Bacillus pseudomonas infantum decreased their production of colonies to some extent after 14 to 21 days, but the number of colonies produced far exceeded the former case and the effect of low temperature exposure was not too great.

(d) The resistance of Staphylococcus aureus to low temperature was the highest elicited. It produced many colonies after 21 days of low temperature exposure almost without any effects.

On the other hand, the controls at room temperature always

produced very [illegible], but in the case of the [illegible] bacteria, which [illegible] to [illegible] or showed better resistance and died faster than those exposed to low temperature. That is, [illegible] a decreased [illegible] after [illegible] days and did not produce a single colony after [illegible] days. [illegible] colonies [illegible] after [illegible] days. [illegible] decreased [illegible] but [illegible] also [illegible] did not produce a single colony after [illegible] days.

[illegible] this article, I should like to extend my [illegible] gratitude to [illegible] for his guidance and his [illegible] criticism. [illegible] I would like to thank Professor [illegible] and [illegible] for their advice and assistance.

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